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TESIS DOCTORAL

Epidemiología molecular de *mycobacterium bovis y mycobacterium caprae* en España

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Sabrina Rodríguez Campos

Directores

Alicia Aranaz Martín
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SABRINA RODRÍGUEZ

CENTRO DE VIGILANCIA SANITARIA VETERINARIA

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CERTIFICAN:

Que la tesis doctoral que lleva por título **"Epidemiología molecular de *Mycobacterium bovis* y *Mycobacterium caprae* en España"** ha sido realizada por la licenciada en Veterinaria Dña. Sabrina Rodríguez Campos en el Departamento de Sanidad Animal de la Facultad de Veterinaria y en el Centro de Vigilancia Sanitaria Veterinaria de la Universidad Complutense bajo nuestra dirección, y estimamos que reúne los requisitos exigidos para optar al título de Doctor Europeo por la Universidad Complutense.

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"Ves, Momo, las cosas son así:
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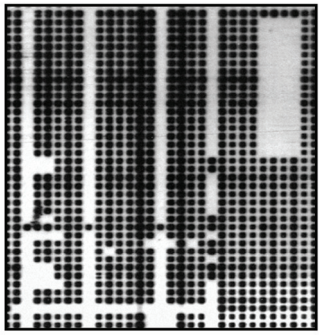
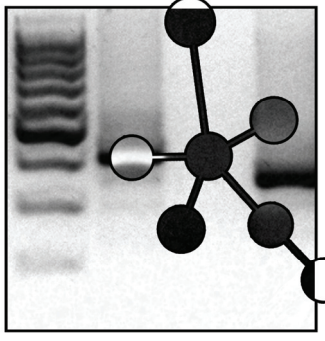
Table of contents

Introduction ... 1
1. Genus *Mycobacterium* ... 3
1.1. General characteristics ... 3
1.2. The mycobacterial cell envelope ... 5
2. The *Mycobacterium tuberculosis* complex ... 7
2.1. *Mycobacterium tuberculosis* ... 12
2.2. *Mycobacterium canettii* ... 13
2.3. *Mycobacterium africanum* ... 14
2.4. Oryx bacillus ... 14
2.5. Dassie bacillus ... 15
2.6. *Mycobacterium pinnipedii* ... 15
2.7. *Mycobacterium microti* ... 16
2.8. *Mycobacterium caprae* ... 16
2.9. *Mycobacterium bovis* ... 17
2.10. *Mycobacterium bovis* BCG ... 19
3. Evolution of the *Mycobacterium tuberculosis* complex ... 21
3.1. General principles of bacterial molecular evolution ... 21
3.2. Diversity among the *M. tuberculosis* complex ... 22
3.3. Phylogeny of the *M. tuberculosis* complex ... 23
3.4. Spread of the *M. tuberculosis* complex ... 29
4. Tuberculosis in animals ... 30
4.1. Relevance of bovine tuberculosis ... 31
4.2. Relevance of caprine tuberculosis ... 34
4.3. Tuberculosis in other animal species ... 36
4.4. Legal framework and economics ... 38
4.5. Pathogenesis ... 40
4.6. Sampling and bacterial identification ... 42
4.6.1. Bacteriological culture ... 42
4.6.2. Molecular identification ... 44
4.7. Zoonotic aspects of *M. bovis* and *M. caprae* ... 45
5. Molecular typing methods ... 48
5.1. Whole genome techniques ... 49
5.1.1. Restriction endonuclease analysis ... 49
5.1.2. Pulsed field gel electrophoresis ... 50
5.1.3. Whole genome sequencing ... 50
5.1.4. Whole genome microarray ... 52
5.2. Partial genome techniques ... 53
5.2.1. Restriction fragment length polymorphism analysis ... 53
5.2.1.1. Insertion sequences IS*6110* and IS*1081* ... 54
5.2.1.2. Polymorphic GC-rich repeat sequences ... 55
5.2.1.3. Typing based on the direct repeat region ... 55
5.2.2. Spoligotyping ... 55
5.2.3. Variable number tandem repeat typing ... 62
5.2.4. IS*6110*-Ampliprinting ... 66
5.2.5. Random amplified polymorphic deoxyribonucleic acid analysis ... 66
5.2.6. Multilocus sequence typing ... 66
5.2.7. RD typing ... 67
5.2.8. Single nucleotide polymorphism typing ... 67
5.3. Molecular epidemiology and databases ... 68

Objectives and organisation of the thesis 71
Chapter I
Molecular demography of *Mycobacterium bovis* and *Mycobacterium caprae* in Spain 79
I.1. High spoligotype diversity within a *M. bovis* population 85
I.2. M. caprae infection in livestock and wildlife 93
I.3. Dendrograms of *M. bovis* and *M. caprae* spoligotypes from Spain 105
I.4 Contributions to conferences and meetings of European projects 109
Chapter II
Molecular typing as a tool in tracking outbreaks caused by *Mycobacterium bovis* 115
II.1 Discrimination of VNTR typing rises with the expansion of a clonal group of *M. bovis* 121
II.2 Limitations of spoligotyping and VNTR typing in a high diversity 145
II.2.1 Tuberculosis in alpacas caused by *M. bovis* 151
II.3 Spoligotyping and MIRU-VNTR typing of *M. bovis* isolates from bullfighting cattle.... 157
II.4 Contributions to conferences and meetings of European projects 163
Chapter III
The Spanish national database of animal tuberculosis - mycoDB.es 169
III.1 A national database of animal tuberculosis - mycoDB.es 173
III.2 Contributions to conferences and meetings of European projects 179
Chapter IV
Phylogeny of *Mycobacterium bovis* in the Iberian Peninsula 185
IV.1. The European 2 clonal complex of *M. bovis* 189
IV.2. The African 2 clonal complex of *M. bovis* 201
IV.3. The European 1 clonal complex of *M. bovis* 211
Discussion 225
Conclusions 237
Summary 241
Resumen en español 245
Objetivos y organización de la tesis 247
Resumen 255
Conclusiones 265
Bibliography 267
Appendix 319
Appendix I - List of abbreviations 321
Appendix II - List of figures 325
Appendix III - List of tables 327
Appendix IV - Sequence submitted to GenBank 329
Appendix V - Review of published spoligotypes 331



Introduction



1. Genus *Mycobacterium*

1.1. General characteristics

The genus *Mycobacterium* (*M.*) belongs to the phylum Actinobacteria, class Actinobacteria, order Actinomycetales, suborder Corynebacterineae, and is given its own family, the *Mycobacteriaceae* (Bergey's Manual of Systematic Bacteriology, 2005). According to "J.P. Euzéby: List of Prokaryotic names with Standing in Nomenclature", which is based on the "Approved Lists of Bacterial Names" (Skerman *et al.*, 1980; Skerman *et al.*, 1989) and subsequent issues of the "International Journal of Systematic Bacteriology" or the "International Journal of Systematic and Evolutionary Microbiology", the genus encompasses 153 validly published species and 11 subspecies (http://www.bacterio.net, updated on 5th August 2011). The genus includes species responsible for a number of serious and widespread human and animal diseases, such as tuberculosis (*Mycobacterium tuberculosis* complex) and leprosy (*Mycobacterium leprae*). The first descriptions of mycobacteria date back to the nineteenth century when Gerhard Henrik Armauer Hansen described the causative agent of leprae *Bacillus leprae* (Hansen, 1874) and Robert Koch described the tuberculous bacillus *Bacterium tuberculosis* (Koch, 1882).

The common characteristics of the genus are weak Gram-positivity, acid-alcohol fastness, slender rod shape, immobility, aerobiosis and the incapability of sporulation. Other minimal standards for assignment of a strain to the genus *Mycobacterium* include DNA G+C content in the range from 61 to 71 mol%, and synthesis of long-chain mycolic acids containing between 60 and 90 carbon atoms which are cleaved to C_{22} to C_{26} fatty acid methyl esters by pyrolysis (Lévy-Frébault and Portaels, 1992). It is now known that pathogenic mycobacteria contain a "capsular" structure that protects the bacteria from microbiocidal activities of the macrophages and also contributes to the permeability barrier of the mycobacterial cell envelope (Daffé and Draper, 1998; Rastogi *et al.*, 2001).

There have been several classifications for species of the genus. Differences in the generation time first led to the division between slow growing and rapid growing mycobacteria. Fast growers form clearly visible colonies within two to seven days, while slow growers have a mean division time of 12-24 hours and therefore require about 15 to 24 days. Fast growers usually are rare pathogens, including the saprophytes. The slow-growers are generally pathogenic for animals and humans and have been subdivided into three groups (Timpe and Runyon, 1954) according to the production of visible carotenoid pigments: photochromogens produce yellow pigmented colonies after exposure to light (group I), scotochromogens produce deep yellow to orange colonies

(group II) and non-chromogens are nonpigmented (group III). Due to practical purposes the genus can also be divided into three groups based on their clinical characteristics: the *M. tuberculosis* complex (MTBC), the group of non-culturable mycobacteria and mycobacteria other than tuberculosis (MOTT), also known as atypical or non-tuberculous mycobacteria (NTM).

Advances in molecular techniques nowadays permit to distinguish the genus *Mycobacterium* using sensitive and rapid techniques based on the amplification of of nucleic acid sequences from the 16S ribosomal ribonucleic acid (16S rRNA) (Böddinghaus *et al.*, 1990; Rogall *et al.*, 1990). rRNA is an essential constituent of bacterial ribosomes that contains stretches of highly conserved sequences and others with some variability and is characteristic for almost every organism (Woese *et al.*, 1987). The 16S rRNA is approximately 1,500 nucleotides in length and is a component of the smaller (30S) subunit of the 70S prokaryotic ribosome. The small but conserved sequence differences in the rRNA molecule allow the definition of specific oligonucleotides in order to differentiate at genus, group, or even species level (Tortoli, 2003). Generally, the slow-growing mycobacteria harbour one copy of the 16S rRNA, except from *M. terrae* (Ninet *et al.*, 1996) and *M. celatum* (Reischl *et al.*, 1998), while fast-growing mycobacteria usually harbour two 16S rRNA fragments, except from *M. chelonae* and *M. abscessus* (Tortoli *et al.*, 2003). The analysis of this molecule has been the most widely used tool for taxonomic studies of the genus (Tortoli, 2006; Biet *et al.*, 2005; Tortoli, 2011; Figure 1).

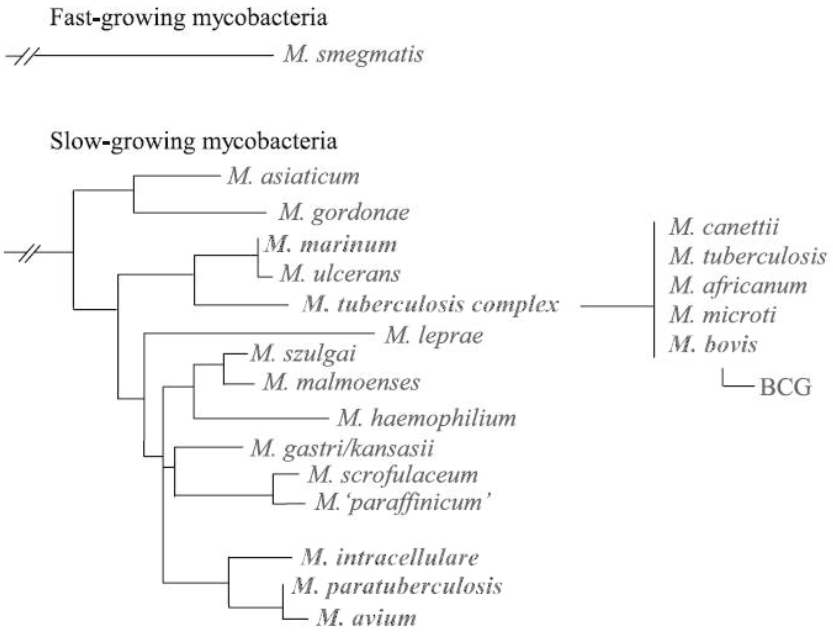


Figure 1. Phylogenetic tree of mycobacteria based on 16S rRNA sequence. Adapted from Biet *et al.*, 2005, with permission from Veterinary Research© http://www.vetres.org/.

The genus *Mycobacterium* exhibits a considerable zoonotic potential encompassing many important human and animal pathogens, some responsible for diseases known since ancient times. Moreover, two members of the *Mycobacterium avium* complex (MAC), *M. avium* subspecies *paratuberculosis* and *M. avium* subspecies *hominissuis*, are considered important zoonotic agents due to the possible implication in Crohn's disease (*M. a. paratuberculosis*) and as opportunistic pathogen in HIV patients (*M. a. hominissuis*) (Inderlied *et al.*, 1993). Other important zoonotic agents are *M. marinum*, *M. fortuitum* and *M. chelonae* that cause piscine mycobacterioses in fish and humans; in humans this mostly occupational disease is also referred to as "fish tank granuloma" or "fish handler's disease" (Decostere *et al.*, 2004). The group of atypical mycobacteria comprises slow-growing species, such as *M. abscessus*, *M. goodii*, *M. wolinskyi* and *M. immunogenum*, as well as fast-growing species, for example *M. genavense*, *M. celatum*, *M. bohemicum* and *M. interjectum*. MOTT are opportunistic pathogens that have gained importance during the last three decades due to the rise of AIDS since they often cause disease in immunocompromised patients. A wide range of pathologies can be caused by MOTT, such as cervical lymphadenitis, posttraumatic and postsurgical infections, chronic lung disease, cutaneous infections or hypersensitivity pneumonitis and the correct species identification is crucial for proper treatment (Tortoli, 2009).

1.2. The mycobacterial cell envelope

The cell envelope made up by the bacterial cytoplasmic membrane, also called inner membrane, the cell wall and the outer layer, called the "capsule" in the case of pathogenic mycobacteria, is essential to enable mycobacteria to survive and grow intracellularly (Daffé and Draper, 1998). Although the constituents of the outer layer are not non-covalently bound to the cell wall and thus are not capsular in a strict sense (Roberts, 1996), it is convenient to refer to it as "capsule" due to its functional implications (Daffé and Etienne, 1999). The cell wall contains three different structures, peptidoglycan, arabinogalactan and mycolic acids, which are covalently linked. Mycolic acids are strongly hydrophobic and are unique to the genera *Mycobacterium* and *Corynebacterium*. Covalent linkage of the mycolic acids leads to a hydrophobic layer, also referred to as the mycomembrane, which forms a lipid shell around the organism. This benefits the members of the MTBC since they are intracellular pathogens and replicate within macrophages, in a hostile environment. The outer part of the mycomembrane contains various free lipids, such as phenolic glycolipids, phthiocerol dimycocerosates, dimycolyltrehalose or cord factor, sulpholipids and phosphatidylinositol mannosides that are intercalated with the mycolic acids. Most of these lipids are specific for mycobacteria. The outer layer is formed by polysaccharides (glucan and arabinomannan) and protein with relatively small quantities of lipid

(Abdallah *et al.*, 2007; Figure 2). In non-pathogenic mycobacteria the outer layer mainly consists of proteins while polysaccharides are more abundant in pathogenic mycobacteria (Daffé and Reyrat, 2008).

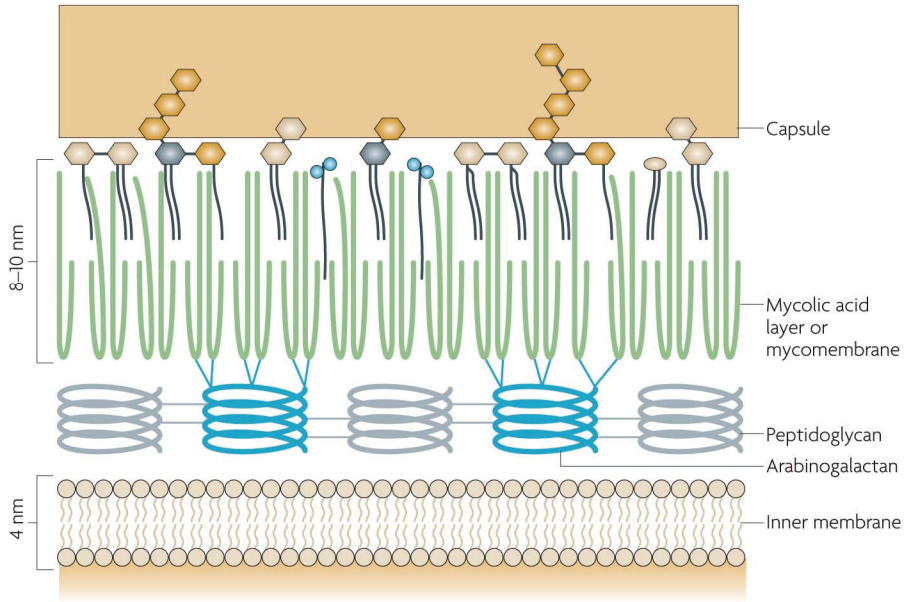


Figure 2. Schematic representation of the cell envelope of *Mycobacterium tuberculosis*. Depicted here is one of the current views of the mycobacterial cell envelope. The cell wall is mainly composed of a large cell-wall core that contains three different covalently linked structures [peptidoglycan (grey), arabinogalactan (blue) and mycolic acids (green)]. From Abdallah *et al.* (2007) with permission from Nature Reviews Microbiology ©.

Due to the high content of lipids, the mycobacteria are robust and have a long survival in the environment. The bacteria resist drying but are killed by sunlight, ultraviolet radiation and pasteurisation (Biberstein and Hirsch, 1999). Various studies have shown that *M. bovis* may have a long survival in the environment (Kelly and Collins, 1978; Morris *et al.*, 1994; O'Reilly and Daborn, 1995; Scanlon and Quinn, 2000), depending on several factors, such as the initial number of bacteria present, the organic matter, pH, temperature, sunlight, humidity and possible interactions with other microorganisms (Scanlon and Quinn, 2000). Several authors suggested that *M. bovis* can be expected to survive up to two years in sub-soil or in slurry-treated soil (Kelly and Collins, 1978; Morris *et al.*, 1994), in faeces up to five months during colder months and less during warmer periods. However, Menzies and Neill (2000) concluded that under natural conditions it appears that survival in the environment is only a few weeks.

In addition to the before mentioned properties, the mycobacterial cell envelope also plays a key role for host–pathogen interactions regarding the *Mycobacterium tuberculosis* complex (MTBC) (Besra and Chatterjee, 1994; Brennan and

Nikaido, 1995; Gordon *et al.*, 2009). Virulence factors implicated in the immunopathogenesis of tuberculosis may represent potential targets for therapeutic or preventive interventions and therefore received special attention during the last years. Certain cell envelope-associated lipids were shown to act as defensive, offensive or adaptive effectors of virulence and thus are able to modulate the immune response in the host (Rastogi *et al.*, 2001; Hotter and Collins, 2011). For example, phthiocerol dimycocerosate (PDIM) is considered to be an important virulence factors since tests with PDIM-deficient mutants yielded strains with attenuated growth in the murine model (Camacho *et al.*, 1999; Cox *et al.*, 1999). The sulpholipids in pathogenic mycobacteria have also been correlated with strain virulence in the guinea pig model (Goren, 1982). The mycolic acid-containing glycolipid, dimycolyltrehalose (TDM), also known as cord factor, has been shown to cause lung granulomas in mice (Yamagami *et al.*, 2001). Much experimental evidence supports the hypothesis that the cell wall lipids are important for virulence, but their effects on host immune response are not entirely disclosed. The pleiotrophic immunological effects of these molecules make it difficult to elucidate the precise mechanism of contribution to the immunopathogenesis of tuberculosis (Karakousis et al, 2004). Moreover, certain key cell wall lipids may only be excreted under certain conditions or during infection which difficults their study (Minnikin *et al.*, 2002). Another capsular substance potentially involved in key steps of pathogenicity is the group of glycans, particularly as they have been shown to mediate the adhesion to and the penetration of bacilli into the host cell (Daffé and Etienne, 1999). For example, lipoarabinomannan (LAM) is a phosphatidylinositol-anchored lipoglycan with diverse biological activities (Hunter and Brennan, 1990). Three classes of LAM have been described in relation to their molecule structure, which are possible virulence factors (Prinzis *et al.*, 1993; Nigou *et al.*, 2003; Guerardel *et al.*, 2002). Novel evidence suggests that mycobacteria dispose of special secretion mechanisms to secrete virulence factors into the extracellular milieu or into the host cell (Abdallah *et al.*, 2007). Ongoing studies of these pathways will hopefully provide new insights into the virulence of the pathogenic mycobacteria.

2. The *Mycobacterium tuberculosis* complex

The mycobacterial species that produce tuberculosis in humans and animals are merged in the *Mycobacterium tuberculosis* complex (MTBC). This group of bacteria is characterized by 99.9% similarity at the nucleotide level and virtually identical 16S rRNA sequences (Böddinghaus *et al.*, 1990, Sreevatsan *et al.*, 1997; Huard *et al.*, 2006). It has a highly clonal population structure with little or no evidence for recombination (exchange of chromosomal DNA) between strains (Supply *et al.*, 2003; Smith *et al.*, 2006b; Hershberg *et al.*, 2009).

To date, the MTBC harbours the following species: *M. tuberculosis* (Koch, 1882), *M. bovis* (Karlson and Lessel, 1970), *M. bovis* BCG (Bacillus Calmette and Guérin, 1921), *M. africanum* (Castets *et al.*, 1968; Castets *et al.*, 1969), *M. microti* (Wells and Oxon, 1937; Reed, 1957), *M. canettii* (van Soolingen *et al.*,1997), *M. pinnipedii* (Cousins *et al.*, 2003), and *M. caprae* (Aranaz *et al.*, 2003); moreover, the Oryx bacillus (Lomme *et al.*, 1976) and the dassie bacillus (Wagner *et al.*, 1958) have been named. The division into different species has a rather epidemiological purpose since it is mainly based on host preference, and it has been suggested that the *M. tuberculosis* complex may instead represent a series of host-adapted groups consistent with the ecotype concept of Cohan (Cohan, 2002; Smith *et al.*, 2006a). Nevertheless, these species, or ecotypes, can be distinguished by their distinct cultural and biochemical characteristics (Collins *et al.*, 1985; Grange *et al.*, 1996) (Table 1).

Table 1. Selected cultural and biochemical characteristics for various species of the *Mycobacterium tuberculosis* complex. +, positive; -, negative; V, variable results; ND, no data available.

Organism and variety	Growth	Colony[a]	Preference of:		Growth in presence of:		Niacin production	Nitrate reduction
			Oxygen[b]	Pyruvate	TCH[c]	PZA[d]		
M. tuberculosis								
Classic	Eugonic	Rough	A	-	+	-	+	+
Asian	Eugonic	Rough	A	-	-	-	+	+
M. africanum								
West African I	Dysgonic	Rough	M	-	-	-	V	-
West African II	Dysgonic	Rough	M	-	-	-	V	-
Dassie bacillus	Dysgonic	Smooth	A	-	-	-	ND	ND
Oryx bacillus	ND	ND	ND	ND	ND	ND	ND	ND
M. pinnipedii	Dysgonic	Rough	A	+	-	-	-**	-
M. microti	Dysgonic	Smooth	A	-	-	-	+	-
M. caprae	Dysgonic	Smooth	M	+	-*	-	-	-
M. bovis	Dysgonic	Smooth	M	+	-	+	-	-
M. bovis BCG	Eugonic	Rough	A	+	-	+	-	-

[a] Colony morphology
[b] Oxygen preference: A= aerobic; M= microaerophilic
[c] TCH: Thiophene- 2- carboxylic acid. *Resistant to 1 and 2 µg TCH/ml, but sensitive to 10 µg TCH/ml.
[d] PZA: Pyrazinamide
** Occasionally weak positive reactions

In addition to the cultural and biochemical features, several molecular characteristics have been described as a useful tool for species differentiation and phylogenetic studies (Table 2). These tests are easy to perform and are less time intensive than the biochemical tests; moreover, they are "binary" in the sense that they gove a yes or no answer. In 1991, a genomic fragment, designated *mtp40*, was described as species specific for *M. tuberculosis* (del Portillo *et al.*, 1991; Parra *et al.*, 1991), although some clinical isolates of *M. tuberculosis* were found to lack this fragment (Liébana *et al.*, 1996). Nowadays, it is known that *mtp40* is encoded by the *plc* gene that lies within a highly polymorphic region present in most, but not all, isolates of *M. tuberculosis*, *M. africanum*, *M. pinnipedii*, and *M. microti*, and is consistently absent from *M. caprae*, *M. bovis* and *M. bovis* BCG isolates (Viana-Niero *et al.*, 2004). In *M. bovis*, *M. bovis* BCG and *M. caprae* the pseudogene *oxyR* (Deretic *et al.*, 1995) has an adenine (A) residue at nucleotide position 285 (Sreevatsan *et al.*, 1996). As a further criterion for the differentiation of *M. bovis*, intrinsic resistance to pyrazinamide (PZA) has been reported (Wayne and Kubica, 1986). This is due to a G substitution at the C residue in codon 57 in the pyrazinamidase gene (*pncA*) (Scorpio and Zhang, 1996). Another G substitution can occur at codon 463 of the catalase-peroxidase gene (*katG*) causing a change of arginine to leucine which is associated with decreased susceptibility to isoniazid (Cockerill *et al.*, 1994; Uhl *et al.*, 1996). A further nucleotide substitution in codon 203 of this gene is able to distinguish between the two subtypes of *M. africanum* type I (Huard *et al.*, 2006). The same study by Huard and colleagues (2006) identified a residue change from C to G at nucleotide 551 of the *mmpL6* gene that differentiates the oryx and dassie bacilli. Further identification can be deduced from the A and B subunits of the DNA gyrase sequence encoded by *gyrA* and *gyrB*, respectively. Codon 95 of the *gyrA* gene displays a change from serine to threonine due to a substitution in a fluoroquinolone resistance-determining region (Takiff *et al.*, 1994). Several nucleotide substitutions have been observed in *gyrB* affecting nucleotide positions 675, 756, 1410 and 1450 (Kasai *et al.*, 2000) and at position 1311 (Niemann *et al.*, 2000b).

Table 2. Overview of selected molecular characteristics for various species of the *Mycobacterium tuberculosis* complex based on the following publications: Parra et al. (1991), Liébana et al. (1996), Espinosa de los Monteros et al. (1998), Kasai et al. (2000), Niemann et al. (2000b), Cousins et al. (2003), Aranaz et al. (2003), Chimara et al. (2004), Viana- Niero et al. (2004), Goh et al. (2006) and Huard et al. (2006). - , negative; V, variable results; ND, no data available.

Organism and variety	Presence of the *mtp40* gene	*oxyR*^{n285} mutation (G to A)[a]	*pncA*^{c57} mutation (CAC to GAC)	*katG* mutation at codon: 203 (ACC to ACT)	*katG* mutation at codon: 463 (CTG to CGG)	*mmpL6*^{n551} mutation (C to G)	*gyrA*^{c95} mutation (AGC to ACC)	*gyrB* mutation at nucleotide 657 (C to T)	*gyrB* mutation at nucleotide 756 (G to A)	*gyrB* mutation at nucleotide 1311 (T to G)	*gyrB* mutation at nucleotide 1410 (C to T)	*gyrB* mutation at nucleotide 1450 (G to T)
M. tuberculosis												
Classic	V	G	CAC	ACC	V	C	V	C	G	T	C	G
Asian	V	G	CAC	ACC	V	ND	-	C	G	T	C	G
M. africanum												
West African I	ND	G	CAC	ND	CTG	C	ACC	C	G	T	C	T
West African II	ND	G	CAC	ND	CTG	C	ACC	C	G	T	C	T
Dassie bacillus	-	G	CAC	ACT	CTG	C	AGC	C	G	T	C	T
Oryx bacillus	ND	G	CAC	ACT	CTG	G	AGC	C	G	T	C	T
M. pinnipedii	V	G	CAC	ACT	CTG	G	ACC	C	G	T	C	T
M. microti	V	G	CAC	ACT	CTG	G	ACC	T	G	T	C	T
M. caprae	-	A	CAC	ACT	CTG	G	ACC	C	A	G	C	T
M. bovis	-	A	GAC	ACT	CTG	G	ACC	C	A	T	T	T
M. bovis BCG	-	A	GAC	ACT	CTG	G	ACC	C	A	T	T	T

[a] The superscript indicates the position of the mutation at either the nucleotide (n) or the codon (c) of the respective genes.

Apart from the before mentioned molecular markers a series of large sequence polymorphisms (LSP), named Regions of Difference (RD), can be used for species differentiation and phylogenetic studies within the MTBC. Three regions (RD1, RD2 and RD3) have been found to be deleted from the vaccine strain *M. bovis* BCG when compared to a virulent *M. bovis* strain (Mahairas *et al.*, 1996) and are assumed to have happened during laboratory culture. Subsequently, the absence of RD4 to RD10 (Gordon *et al.*, 1999) and RD11 to RD13 (Brosch *et al.*, 2002) was described for all *M. bovis* isolates (see section 3.3. and 5.2.7.).

2.1. *Mycobacterium tuberculosis*

M. tuberculosis is the aetiological agent of human tuberculosis and is the most important bacterial pathogen of humans, with approximately one third of the global population infected and causing up to two million deaths annually. *M. tuberculosis* has also been isolated from cattle (Prasad *et al.*, 2005; Srivastava *et al.*, 2008; Fetene *et al.*, 2011; Ameni *et al.*, 2011; Romero *et al.*, 2011), goats (Cadmus *et al.*, 2009; Hiko and Agga, 2011), domestic pigs (*Sus scrofa domestica*) (Mohamed *et al.*, 2009; Jenkins *et al.*, 2011), cats (*Felis silvestris catus*) and dogs (*Canis lupus familiaris*) (Clercx *et al.*, 1992; Aranaz *et al.*, 1996b; Erwin *et al.*, 2004; Parsons *et al.*, 2008), birds (Hoop *et al.*, 1996; Schmidt *et al.*, 2008) and also from several wildlife species including zoo animals (Montali *et al.*, 2001; Alexander *et al.*, 2002; Une and Mori, 2007; van Helden *et al.*, 2009; Angkawanish *et al.*, 2010). On Middlebrook, an agar based culture medium, or Löwenstein-Jensen (LJ), an egg based culture medium, it takes four to six weeks to obtain visible small and buff coloured colonies. The colonies have a patterned texture due to the tight cording of the cells (Runyon, 1970). This feature can be used to distinguish *M. tuberculosis* from other mycobacterial species. Before molecular techniques were available the differentiation between *M. tuberculosis* and *M. bovis* was a tedious procedure based on growth and biochemical characteristics including oxygen preference, reduction of nitrates, nicotinamidase and pyrazinamidase activity and could last more than three months.

The whole genome sequence of *M. tuberculosis* was published in 1998 (Cole et. al., 1998; Camus *et al.*, 2002) (Figure 3). Since then, there has been an intensification of activities in the field of molecular tuberculosis research, especially in the areas of comparative genomics, functional genomics and envelope biogenesis.

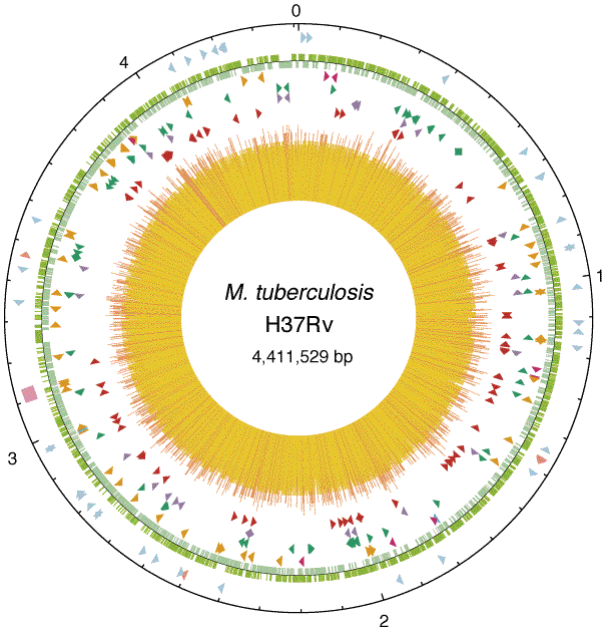


Figure 3. Circular map of the chromosome of *Mycobacterium tuberculosis* H37Rv. The outer circle shows the scale in Mb, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, others are pink) and the direct repeat region (pink cube); the second ring inwards shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (centre) represents G+C content, with 65% G+C in yellow, and 65% G+C in red. From Cole *et al.* (1998) with permission from Nature©.

2.2. *Mycobacterium canettii*

M. canettii is the most divergent subspecies within the complex, exhibiting a smooth and glossy colony morphology and a rapid growth in vitro. It was first mentioned by the French microbiologist Georges Canetti in 1969 and preserved and studied extensively at the Pasteur Institute (Daffé *et al.*, 1987; Daffé *et al.*, 1991). Van Soolingen and colleagues described *M. canettii* isolated from a Somali child as a novel pathogenic taxon of the MTBC in 1997 (van Soolingen *et al.*, 1997). It has also been isolated from a 56-year-old Swiss man with abdominal lymphatic tuberculosis who lived in Kenya (Pfyffer *et al.*, 1998) and from two soldiers of the French Foreign Legion in Djibouti (Miltgen *et al.*, 2002). Tuberculosis caused by *M. canettii* appears to be an emerging disease in the Horn of Africa. However, the natural reservoir and host range of this pathogen are still unknown. *M. canettii* is nowadays considered an outgroup of the MTBC due to its divergence from all other members of the complex and the evidence for recombination (Gutiérrez *et al.*, 2005), however the status of *M. canettii* as an outgroup to the MTBC complex has been challenged (Smith *et al.*, 2009a).

2.3. *Mycobacterium africanum*

This species was first described as tuberculosis bacilli of the African type in Dakar, Senegal, in 1968 (Castets *et al.*, 1968). The strains were described as intermediate between *M. tuberculosis* and *M. bovis*. Nowadays, this pathogen causes half of the human tuberculosis cases in West Africa (Kallenius *et al.*, 1999; de Jong *et al.*, 2009). A slower clinical progression has been observed in patients infected with *M. africanum* (de Jong *et al.*, 2008). Furthermore, it has been suggested that a specific host-pathogen adaptation to humans with a defined host genetic predisposition has occurred (Gagneux *et al.*, 2006; Intemann *et al.*, 2009). This mycobacterial species was first divided into two types, I and II, based on cultural, biochemical and molecular characteristics (Tables 1 and 2), but in 2004 *M. africanum* type II was reclassified into *M. tuberculosis* sensu stricto (Mostowy *et al.*, 2004b), while *M. africanum* type I was subdivided into West African I, prevalent around the Gulf of Guinea, and West African II prevalent in western West Africa (Gagneux *et al.*, 2006; de Jong *et al.*, 2010). *M. africanum* has sporadically been reported from patients outside of West Africa, including Germany (Meissner *et al.*, 1969; Jungbluth *et al.*, 1978; Schröder *et al.*, 1982), the United Kingdom (UK) (Grange and Yates, 1989), France (Frottier *et al.*, 1990), the United States of America (USA) (Desmond *et al.*, 2004), Kazakhstan (Demkin et la., 2008) and Spain (Pérez de Pedro *et al.*, 2008).

The genetic proximity of *M. africanum* to the animal isolates raises the suspicion of a zoonotic reservoir. Although this pathogen has been isolated from monkeys (Thorel, 1980) and cows (Cadmus *et al.*, 2006; Rahim *et al.*, 2007; Cadmus *et al.*, 2010), these findings were sporadic and could not substantiate this suspicion.

2.4. Oryx bacillus

The MTBC includes two strains, the oryx and dassie bacilli, which have not been given a species name but are named after the host from which they are usually isolated. These members were considered a variant of *M. bovis* in earlier studies, but have later been found to have distinct molecular features (Table 2) that distinguish them from *M. bovis* (Huard *et al.*, 2006). The oryx bacillus has first been described in 1976 as the causative agent of tuberculosis in two East African oryxes (*Oryx beisa*) (Lomme *et al.*, 1976). The oryx bacillus has been recovered in few occasions from antelope species like waterbuck (*Kobus ellipsiprymnus*) and Arabian oryx (*Oryx leucoryx*) (van Soolingen *et al.*, 1994; Greth *et al.*, 1994) and from free-ranging buffalo (*Syncerus caffer*) (van Helden *et al.*, 2009). Recently, a novel genetic marker, a serine to alanine mutation at codon 38 in gene *Rv2042c*, has provided evidence for transmission to humans suggesting that its host range is broader than previously thought (van Ingen *et al.*, 2010).

Smith and colleagues (2006a) showed that a number of isolates, including the

oryx strain had distinct characteristics and could be considered as another species or ecotype within the MTBC. They named this new clade as *M. bovis* (antelope) (Smith *et al.*, 2006a). Strains of *M. bovis* (antelope) have been isolated from females in Australia of Indian and Bangladeshi origin (N. H. Smith, personal communication).

2.5. Dassie bacillus

The dassie bacillus, like the oryx bacillus, is named after its most common host, the hyrax or dassie (*Procavia capensis*) found in South Africa and the Middle East. The first report dates back to the late 1950s (Wagner *et al.*, 1958). Growth characteristics are similar to *M. microti* (Smith, 1960) and it was not until 2004 that a molecular marker specific for the dassie bacillus was described (Mostowy *et al.*, 2004a). The dassie bacillus has been isolated from captive hyrax imported from South Africa in Australia (Cousins *et al.*, 1994), from a meerkat (*Suricata suricatta*) in a Swedish zoo (Mostowy *et al.*, 2002) and from wild hyrax from South Africa (Parsons *et al.*, 2008a; van Helden *et al.*, 2009). In early years, reduced virulence in guinea pigs and rabbits was demonstrated (Smith, 1965) and due to the high similarity to *M. microti* it has often been discussed if the dassie bacillus is a variant of this vole bacillus. Possible reasons for the attenuation remain speculative (Mostowy *et al.*, 2002; Pym *et al.*, 2002; Lewis *et al.*, 2003) and might not be revealed until the completion of the genome sequence (http://www.sanger.ac.uk/resources/downloads/bacteria/mycobacterium.html). The dassie bacillus has been linked to *M. africanum* because of coincident polymorphisms; therefore, these species might have close evolutionary ties (Huard *et al.*, 2006).

A similar pathogen, proposed to be named *M. mungi* sp. nov., which presents characteristics of the dassie bacillus has been isolated from banded mongooses (*Mungos mungo*) in Botswana and a LSP and SNP have been evaluated for its differentiation from the dassie bacillus (Alexander *et al.*, 2010).

2.6. *Mycobacterium pinnipedii*

M. pinnipedii, formerly known as “seal bacillus” due to the host species from which it was first isolated (Forshaw and Phelps, 1991; Cousins *et al.*, 1993), has been classified as a separate species of the MTBC based on its molecular characteristics (Table 2) (Cousins *et al.*, 2003). Its natural host are seal (pinniped) species; *M. pinnipedii* has been reported from captive and wild Australian sea lions (*Neophoca cinerea*), Australian fur seal (*Arctocephalus pusillus doriferus*) (Forshaw and Phelps, 1991; Cousins *et al.*, 1993; Woods *et al.*, 1995) and New Zealand fur seals (*Arctocephalus forsteri*) (Hunter *et al.*, 1998). It has also been isolated from a captive Southern sea lion (*Otaria flavescencs*), wild South American fur seals (*Arctocephalus australis*) and a wild Subantarctic fur seal (*Arctocephalus tropicalis*) in Uruguay and Argentina (Bernardelli *et al.*, 1994, Bastida *et*

al., 1999). Furthermore a Brazilian tapir (*Tapirus terrestris*) and South American fur seals from a zoo in Great Britain were found to be infected (Cousins *et al.*, 2003). Nevertheless it is capable of infecting other mammalian animal species like dolphins (*Delphinidae*), llama (*Lama glama*), and gorilla (*Gorilla sp.*) (Australian Wildlife Health Network, 2010). Seal trainers who worked with affected seal colonies in Australia and the Netherlands were also found infected with *M. microti* confirming the zoonotic potential of this pathogen (Thompson *et al.*, 1993; Kiers *et al.*, 2008).

2.7. *Mycobacterium microti*

M. microti had been originally identified as a pathogen of small rodents in Great Britain (Wells, 1937 and 1946) where this form of tuberculosis was found in voles (*Microtus agrestis*), bank voles (*Myodes glareolus*), wood mice (*Apodemus sylvaticus*) and shrews (*Sorex araneus*). This pathogen has also been isolated from other mammalian species like cats (*Felis catus*) (Gunn-Moore *et al.*, 1996; Kremer *et al.*, 1998), llamas (*Lama vicugna*), alpaca (*Lama pacos*), tapir (*Tapirus sp.*) and badgers (*Meles meles*), (Pattyn *et al.*, 1970; van Soolingen, 1998a; Smith *et al.*, 2009b; Rüfenacht *et al.*, 2011).

In the 1950s, *M. microti* was used as live vaccine in former Czechoslovakia (now Czech Republic and Slovakia), the UK and former Rhodesia (now Zimbabwe) (Paul, 1961; Sula and Radkovsky, 1976; Hart and Sutherland, 1977). Both attenuated and non-attenuated vaccines seemed safe and effective, although no more effective than vaccination with the commonly used *M. bovis* BCG. Evidence for protection against *M. bovis* in *M. microti* vaccinated cattle had been recorded in early years (Wells, 1949). More recently, it has also been described as effective in laboratory mice (Manabe *et al.*, 2002) and has been suggested as vaccine for badgers (*Meles meles*) in the UK (Jones, 2010). However, *M. microti* was found to produce infections in immunocompromised and immunocompetent humans from The Netherlands (van Soolingen *et al.*, 1998b), Germany (Niemann *et al.*, 2000a), Switzerland (Cavanagh *et al.*, 2002), England and Scotland (Xavier Emmanuel *et al.*, 2007) casting doubts on the safety of the *M. microti* vaccine. Smith and colleagues (2009b) suggested that the restricted geographical localisation of *M. bovis* in cattle in Great Britain might be explained if badgers, the maintenance host for *M. bovis* in Great Britain, are naturally vaccinated by exposure to *M. microti* in areas with infected voles.

2.8. *Mycobacterium caprae*

Mycobacterium caprae (Aranaz *et al.*, 2003), formerly known as *M. tuberculosis* subsp. *caprae* (Aranaz *et al.*, 1999) and *M. bovis* subsp. *caprae* (Niemann *et al.*, 2002), forms a genetically distinct cluster within the MTBC. The main features differentiating these isolates from the other members are a special combination of polymorphisms in

the pseudogene (*oxyR*) and the pyrazinamidase (*pncA*), catalase (*katG*), and gyrase (*gyrA* and *gyrβ*) genes (Table 2).

This pathogen has been recognised in many Central and Western European countries, where it has been occasionally isolated from cattle (*Bos taurus*) (Pavlik *et al.*, 2002a; Pavlik *et al.*, 2002b; Erler *et al.*, 2004; Prodinger *et al.*, 2005; Duarte *et al.*, 2008; Boniotti *et al.*, 2009; Cunha *et al.*, 2011b), domestic pig (*Sus scrofa domestica*) (Pavlik *et al.*, 2002a), wild and farmed red deer (*Cervus elaphus*) (Pavlik *et al.*, 2002a; Prodinger *et al.*, 2002), wild boar (*Sus scrofa*) (Machackova *et al.*, 2003; Erler *et al.*, 2004; Cunha *et al.*, 2011b), and the zoo animals Siberian tiger (*Panthera tigris altaica*) (Lantos *et al.*, 2003), bactrian camel (*Camelus ferus*) (Erler *et al.*, 2004), dromedary camel (*C. dromedarius*) and bison (*Bison bison*) (Pate *et al.*, 2006). In Spain, *M. caprae* was classified as *M. bovis* in earlier years (Aranaz *et al.*, 1996) but later on described as the main aetiological agent of caprine tuberculosis (Aranaz *et al.*, 1999). Subsequently, it has been reported from a small number of wild boar (Parra *et al.*, 2003; Aranaz *et al.*, 2004; Gortázar *et al.*, 2005) and also from sheep (Muñoz Mendoza *et al.*, 2011). Regarding cattle-to-cattle transmission, the same pattern was found in isolates from different farms in the same area indicating transmission of the pathogen between neighbouring farms. Also, the finding of the same genotype in cattle and wild boar of the same district in Hungary (Erler *et al.*, 2004), cattle and pig in a same farm (Pavlik *et al.*, 2002), and amongst zoo camel and bison (Pate *et al.*, 2006) indicated transmission between these animal species although in these cases a common infection from a different source could not be ruled out.

2.9. *Mycobacterium bovis*

M. bovis is the main aetiological agent of bovine tuberculosis, but is known to have the broadest host range among the MTBC members. In 1898, the pathologist Theobald Smith showed that tubercle bacilli isolated from humans and those isolated from cattle differed in their ability to infect different animal species (Smith, 1898); in spite of the general acceptance of *M. bovis* as different from *M. tuberculosis*, it was not until 1970 that *M. bovis* was officially recognized as a species (Karlson and Lessel, 1970).

The main animal host of *M. bovis* are species of the family *Bovidae*, including the subfamilies *Bovinae* (bovids) and *Caprinae* (caprids). The pathogen has been most often isolated from domestic cattle (*Bos taurus*) but also from several other species of the family *Bovidae*, including several species of bison, buffalo and antelope (Clancey *et al.*, 1977; Bengis *et al.*, 1996; Radunz, 2006; Wobeser, 2009; de Garine-Wichatitsky *et al.*, 2010; Himsworth *et al.*, 2010), topis (*Damaliscus korrigum*), kudus (*Tragelaphus* sp.) (Cleaveland *et al.*, 2005), goats (*Capra aegagrus hircus*) (Cousins, 2001; Crawshaw *et al.*, 2008; Cadmus *et al.*, 2009; Daniel *et al.*, 2009; Hiko and Agga, 2011), sheep (*Ovis aries*)

(Mallone *et al.*, 2003; Muñoz Mendoza *et al.*, 2011) and chamois (*Rupicapra rupicapra*) (Bouvier, 1951). Generally, domestic cattle are the most affected species although some authors speculated about possible differences in susceptibility between different cattle species (*Bos taurus* and *Bos indicus*) (Ameni *et al.*, 2007a). *M. bovis* can also cause disease in members of the family *Suidae* including domestic pigs and wild boar (*Sus scrofa*) (Parra *et al.*, 2003; Aranaz *et al.*, 2004a; Gortázar *et al.*, 2005; Schmidbauer *et al.*, 2007; Jenkins *et al.*, 2011; Barandiaran *et al.*, 2011). Isolations from horses (*Equus ferus caballus*) have also been reported (Monreal *et al.*, 2001; Keck *et al.*, 2010), as well as from dogs (Ellis *et al.*, 2006; Shrikrishna *et al.*, 2009) and cats (Aranaz *et al.*, 1996b; Monies *et al.*, 2000). In recent years, *M. bovis* infection in camelids (*Camelidae*) (Veen *et al.*, 1991; Barlow *et al.*, 1999; Twomey *et al.*, 2007; Ryan *et al.*, 2008; Twomey *et al.*, 2009; Mamo *et al.*, 2010) has caused polemic due to the difficulties of the diagnosis with traditional techniques (Buick, 2006; Álvarez *et al.*, 2011). Furthermore, *M. bovis* has been found in various wildlife species like red deer (*Cervus elaphus*), fallow deer (*Dama dama*), elk (*Cervus canadensis*), wild boar, foxes (*Vulpes vulpes*), badgers, Iberian lynxes (*Lynx pardinus*), hares (*Lepus sp.*), rabbits (*Leporidae*), hedgehogs (*Erinaceidae*), brushtail possums (*Trichosurus vulpecula*), capybaras (*Hydrochoerus hydrochaeris*), lions (*Panthera leo*) and primates (Gallagher and Clifton-Hadley, 2000; Coleman and Cooke, 2001; Pavlik *et al.*, 2002a; Aranaz *et al.*, 2004a; Corner, 2006; Ryan *et al.*, 2006; Une and Mori, 2007; O'Brien *et al.*, 2008; Balseiro *et al.*, 2011). Human beings are also susceptible to *M. bovis* due to its zoonotic potential (Cosivi *et al.*, 1998; Ojo *et al.*, 2008; Rodríguez *et al.*, 2009; Michel *et al.*, 2010; Jenkins *et al.*, 2011).

In 2003, the first whole genome sequence of a *M. bovis* strain was completed (Figure 4; Garnier *et al.*, 2003) and found to be much smaller than the *M. tuberculosis* genome (Cole *et al.*, 1998). This suggests that *M. bovis* evolved from an *M. tuberculosis*-like organism.

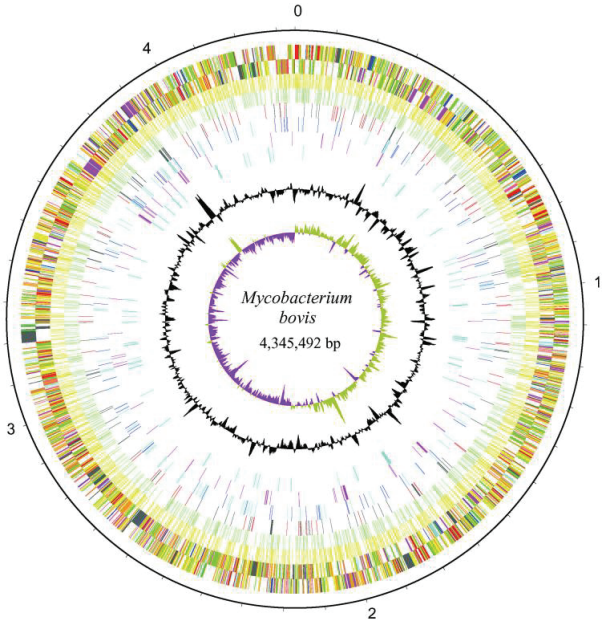


Figure 4. Circular representation of the *Mycobacterium bovis* genome. The scale is shown in megabases by the outer black circle. Moving in from the outside, the next two circles show forward and reverse strand CDS, respectively, with colors representing the functional classification. Comparisons with the *M. tuberculosis* H37Rv sequence are then shown, with transitions (yellow) and transversions (green), then insertions (red, 1 bp; black _1 bp) and deletions (dark blue, 1 bp; light blue, 1 bp); sequence replacements by novel regions in *M. bovis* are then shown (purple). IS elements and phage (cyan) are displayed in the following circle, with G+C content and then finally GC bias (G+C)/(G+C) shown by using a 20-kb window. From Garnier *et al.* (2003) with permission from PNAS © (2003) National Academy of Sciences, U.S.A.

2.10. *Mycobacterium bovis* BCG

The Bacille Calmette Guérin (BCG) is since 1921 the only vaccine available for tuberculosis today and is therefore one of the oldest vaccines, the most commonly used on a global scale, as well as one of the most controversial. It was derived by *in-vitro* attenuation of a *M. bovis* strain by subculturing the strain every three weeks over a long period on bile-glycerol-potato medium. This derivation was result of pathogenesis experiments by Léon C.A. Calmette and Camille Guérin at the Institut Pasteur (Lille, France). A total of 230 passages were performed over 13 years before the organism was found safe in guinea pigs (*Cava porcellus*), cows, horses, hamsters, mice and rabbits and provided protective immunity to challenge with virulent *M. tuberculosis* (Guérin and Rosenthal, 1957). The attenuated strain has been maintained by continuous subculture over decades which led to genetic polymorphism in this strain and therefore a variety of *M. bovis* BCG strains (Behr and Small, 1999; Garcia-Pelayo *et al.*, 2009) (Figure 5).

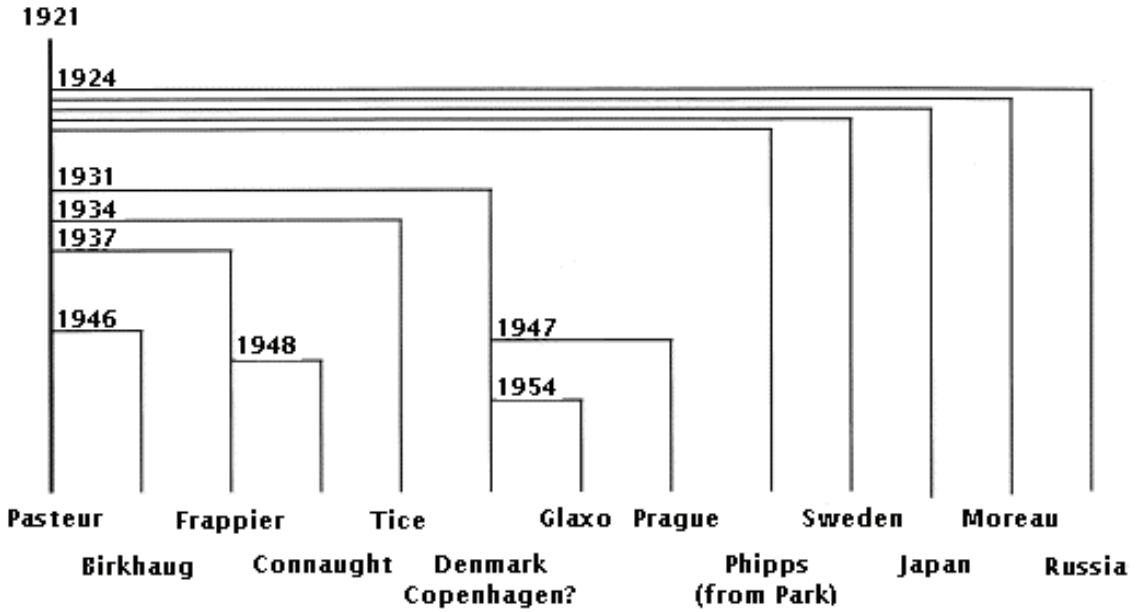


Figure 5. Genealogy of *Mycobacterium bovis* BCG strain dissemination. Vertical axis scales to time. Horizontal dimension does not scale to genetic difference. From Behr and Small, 1999 with permission from Elsevier©.

Today, the organism is maintained using a seed lot production technique to limit further genetic variation using lyophilized cells. BCG is widely given newborns in developing countries and also in countries where tuberculosis is considered endemic. It is most effective in protecting children from the disease. Today, it is estimated that more than 1 billion people have received BCG. In Spain, the BCG vaccine was administered until the late 1970s. The safety of this vaccine has not been a serious issue until recently. There is a concern that the use of the vaccine in persons who are immunocompromised may result in infection caused by the live BCG itself (Mignard *et al.*, 2006; World Health Organization http://www.who.int/vaccine_research/diseases/tb/vaccine_development/bcg/en/).

A different application of BCG is its use for the treatment of bladder cancer by intravesical administration. Immunotherapy with BCG has remained the most effective local therapy against this human neoplasm for the last three decades (Herr and Morales, 2008; Zieger and Jensen, 2011).

3. Evolution of the *Mycobacterium tuberculosis* complex

Reports on clinical symptoms indicating tuberculosis, formerly called phtisis, date back to scripts from Ancient Greece by Herodotus and Aretaeus, from the Roman Empire by Pliny the Younger and to the Hindu Upanishads (*ca.* 1.500 BC). Tuberculous lesions have been found in mummies from Ancient Egypt and Neolithical settlements (Morse *et al.*, 1964; Zimmermann, 1979; Nerlich *et al.*, 1997; Zink *et al.*, 2005; Hershkovitz *et al.*, 2008) suggesting the presence of mycobacteria in ancient civilizations. An archaeological study describing the attempt of spoligotyping of recovered DNA of an extinct long-horned bison from the late Pleistocene with pathological alterations suggestive of tuberculosis hints at an even earlier (17,000 BP) existence of a causative agent of tuberculosis (Rothschild *et al.*, 2001). However, molecular studies on ancient DNA should be interpreted carefully, since degradation and modification of the DNA by progressive oxidative damage and the high risk of contamination handicap the analysis of ancient microbes (Zink *et al.*, 2002; Donoghue *et al.*, 2004). It was long believed that *M. tuberculosis* had evolved from cattle adapted *M. bovis* to become a human adapted pathogen, and it was not until the completion of the whole genome sequences of *M. tuberculosis* H37Rv (Cole *et al.*, 1998) and *M. bovis* AF2122/97 (Garnier *et al.*, 2003) that this hypothesis could be disproved.

3.1. General principles of bacterial molecular evolution

Studies of evolutionary history contribute to the understanding of contemporary disease by analysing pathogen occurrence, frequency and host-pathogen interaction in historic times and populations. Biological evolution is based on the three pillars (i) genetic variation, (ii) natural selection and (iii) reproductive or geographic isolation (Arber, 2003). (i) Spontaneous sequence alterations (mutations) in the genome of an organism are a prerequisite for biological evolution, thus genetic variation is the base material for evolution. Such *de novo* mutations can be spread by vertical transmission onto the descendants of a cell or by horizontal exchange of genetic material between unrelated cells. (ii) Natural selection refers to the differential reproduction of cells dependent on their genetic makeup in response to the environment, and therefore determines the evolutionary directions taken by the organism in question. However, modern studies of bacterial population genetics show that neutral influences can be as powerful as natural selection in determining the evolutionary trajectory of bacterial species, especially in small populations. (iii) Isolation influences the evolutionary process, in the case of reproductive isolation by reducing the fertility between related organisms or by giving way for several groups of related organisms to evolve independently by geographic isolation. Thus, isolation can lead to speciation of organisms.

As smaller chromosomes can replicate faster which results in faster growth and better chances to out-populate bacteria with larger genomes, there is a constant evolutionary pressure on bacteria towards a smaller genome. This process occurs extremely slowly, since a balance is needed between the decrease of genome size and the protection from becoming extinct by degeneration of the genome (Maniloff, 1996). Evolutionary events such as population bottlenecks (or genetic bottleneck) strongly influence the evolution of a group of organisms such as the MTBC. A population bottleneck describes a significant reduction in the size of a population that causes the extinction of many genetic lineages within that population and therefore reduction of the genetic diversity. The eradication programmes for bovine tuberculosis represent such a bottleneck and its effect has been extensively studied in the British Isles, where very low incidences of bovine tuberculosis were achieved from the 1960s until the mid-1980s but then the disease gradually regressed (Smith *et al.*, 2006b). A different sort of genetic bottleneck is the Founder effect which describes the start of a new population by only few members of the original population and leads to reduced genetic variation. A severe reduction in diversity can also be caused by a selective sweep due to an entire chromosome hitch-hiking to fixation as a highly selected locus drives the chromosome to fixation (Maynard Smith and Haigh, 1974). Genome reduction might also have led to the pathogenicity of the slow-growing mycobacteria, since they seem to have evolved from the generally harmless fast-growing ancestors (Rogall *et al.*, 1990; Smith *et al.*, 2009a).

Genetic variation analysis or genotyping is used to track different strains in order to gain information on patterns of spread, infectivity and pathogenicity. Since the various elements used as markers have different rates of mutation, they can be used to assess changes of either shorter or longer periods of time. Markers with a high mutation rate are required for disease tracking while markers with a slow molecular clock can be used to monitor evolution over tens of thousands of years. It is important to understand the patterns of variation and evolution of different molecular markers to obtain an insight into their usefulness for different applications (Arnold, 2007).

3.2. Diversity among the *M. tuberculosis* complex

The MTBC is believed to be a strictly clonal group of organisms, which means that recombination is rare or absent (Supply *et al.*, 2003; Smith *et al.*, 2006b; Hershberg *et al.*, 2009). Only a limited number of genes have been reported that have possibly been acquired by horizontal gene transfer (Martín *et al.*, 1990; Kinsella *et al.*, 2003; Blanc-Potard and Lafay, 2003; Rosas-Magallanes *et al.*, 2006) and recombination has only been observed in *M. canettii* (Gutiérrez *et al.*, 2005). In the absence of horizontal genetic exchange, a specific mutation will be present in all descendants of the strain in which it

arose and will furthermore be in linkage with mutations that have emerged during the evolution of a lineage. Consequently the population will be in linkage disequilibrium, in contrast to populations in severe linkage equilibrium where mutations in different sites occur randomly (Maynard Smith *et al.*, 1993; Spratt and Maiden, 1999).

Genetic diversity among isolates of the MTBC may in great measure be caused by large sequence polymorphisms, such as RDs (Mahairas *et al.*, 1996; Brosch et al, 1998; Behr *et al.*, 1999; Gordon *et al.*, 1999) (see section 2.). Since these genetic deletions are expected to represent unidirectional genetic events, their distribution is highly suggestive of a phylogeny for the MTBC (Kato-Maeda *et al.*, 2001; Mostowy *et al.*, 2002; Brosch *et al.*, 2002; Huard *et al.*, 2003). In addition, samples that present a greater number of RD deletions are, as expected in a clonal organism, missing all the same regions that are absent from samples with fewer RD deletions (Mostowy *et al.*, 2002).

The single nucleotide polymorphisms (SNPs) that differentiate between the different species have been discussed above (Table 2). Many SNPs have been shown to be involved in drug resistance and virulence, for example mutation in *pncA* (Scorpio and Zhang, 1996), *phoP* (Pérez *et al.*, 2001) or *phoT* (Collins *et al.*, 2003). Musser (1995) theorised that the limited number of synonymous (silent) nucleotide substitutions in structural genes found in the MTBC indicates that the even more limited events of amino acid polymorphism (non-synonymous SNP) most often have functional implications. Synonymous SNPs have been found to have functional consequences in rare cases (Kimchi-Sarfaty *et al.*, 2007). Furthermore, it was stated that the lack of neutral mutations in structural genes might also hint at an evolutionary recent global dissemination of the MTBC (Sreevatsan *et al.*, 1997) which supposedly took place after undergoing a bottleneck at the time of speciation about 15,000 to 20,000 years ago (Kapur *et al.*, 1994). However, this simplistic explanation for the lack of diversity in the MTBC has been challenged by Smith and colleagues (2009b). The use of SNPs to establish phylogenies is increasing and different sets of SNPs have been recently analysed within members of *M. tuberculosis* (Gutierrez *et al.*, 2005; Filliol *et al.*, 2006; Gagneux and Small, 2007; Hershberg *et al.*, 2008; Abadia *et al.*, 2010; Schürch *et al.*, 2011) and *M. bovis* (Smith *et al.*, 2006b; Garcia-Pelayo *et al.*, 2009).

3.3. Phylogeny of the *M. tuberculosis* complex

Large genomic deletions, named RDs in the MTBC, are widely used for the construction of phylogenies due to their low mutation rate (Brosch *et al.*, 2002; Smith *et al.*, 2006b; Gagneux and Small, 2007), because they are less likely to be homoplastic than SNPs and are easy to analyse (PCR). Sequence analysis of the RD deletions confirmed that these represent the same genetic event, because the flanking sequences observed are identical for all isolates. In this regard, RD6 is an exception because it is a highly

repetitive region; therefore the exact site of RD6 could not be confirmed. Mostowy and colleagues (2002) did not assign the same level of confidence that absence of RD6 consistently represents the same large sequence polymorphism. Comparative genome analysis showed that it was unlikely that *M. tuberculosis* evolved from *M. bovis*. The fact that the open reading frame (ORF) structures at junction points are truncated supplies compelling evidence that RDs are deletions from the *M. bovis* genome rather than insertions into the *M. tuberculosis* genome (Gordon *et al.*, 1999; Gordon *et al.*, 2001). Insertion of a polygenic region into the *M. bovis* genome which causes the completion of an existing truncated ORF, seems far less likely than the disruption of that ORF during a deletion event. Although selective pressure could theoretically lead to convergence by deleting the same genomic region in unrelated strains, this is highly unlikely because independent deletion would not be expected at exactly the same base pair and also because the RDs are not flanked by repetitive sequences that are prone to generate identical deletions more frequently (Brosch *et al.*, 2002). Empirically, such a deletion convergence, named homoplasy, has not been observed in samples studied to date (Brosch *et al.*, 2000; N. H. Smith, personal communication). The most universally accepted description of the phylogeny of the MTBC is based on the analysis of RDs and a selection of SNPs (Brosch *et al.*, 2002). This phylogeny suggests that *M. tuberculosis*-like organism is the ancestor of all members of the MTBC, except from *M. canettii*, and *M. bovis* the farthest descended species (Figure 6).

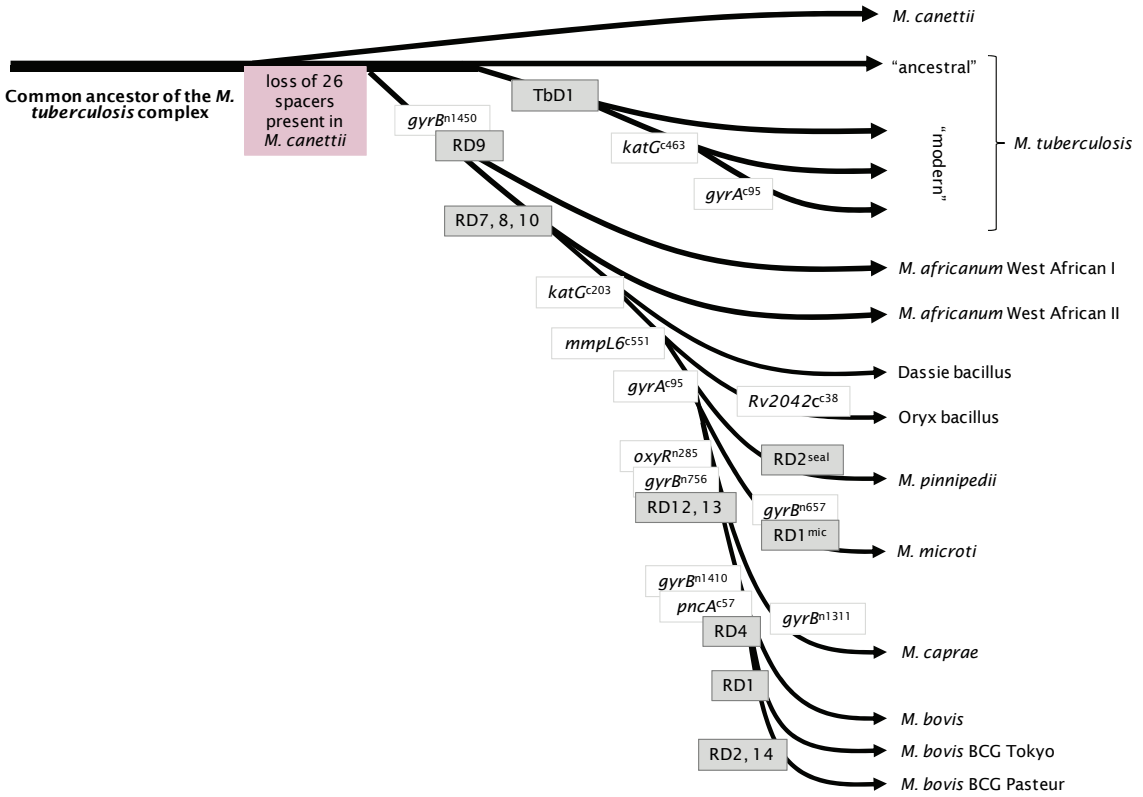
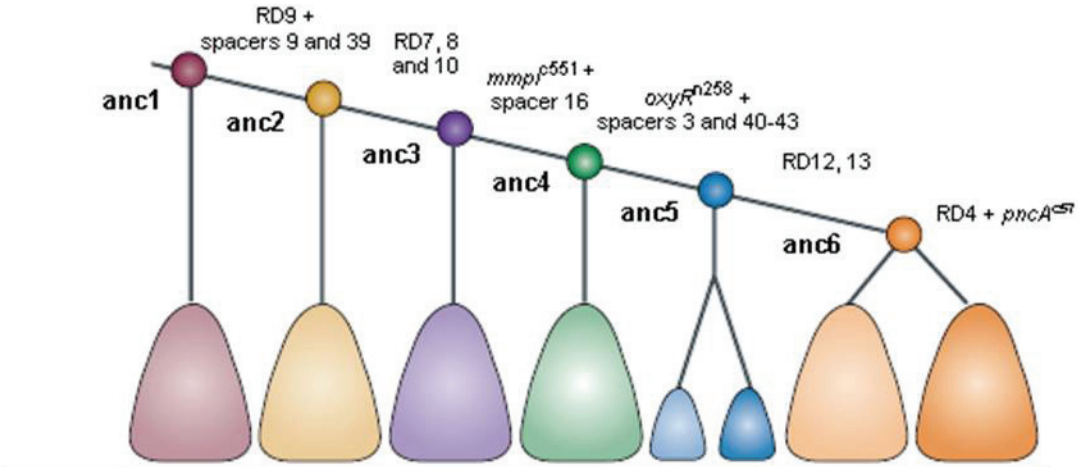


Figure 6. Evolution of the *Mycobacterium tuberculosis* complex based on the presence/absence of regions of difference (RDs) and single nucleotide polymorphisms (SNPs). Grey boxes indicate the loss of a region of difference (RD). White boxes indicate SNPs; superscripts mark the position of the mutation at either the nucleotide (n) or the codon (c) of the respective genes. Adapted from Brosch *et al.* (2002), including information from Kasai *et al.* (2000), Niemann *et al.* (2000b), Cousins *et al.* (2003), Bigi *et al.* (2005), Huard *et al.*, (2006), Smith *et al.* (2009b), de Jong *et al.* (2010) and van Ingen *et al.* (2010).

Smith and colleagues (2006b) have extended the evolutionary scenario of Brosch and colleagues (2002) and showed that the loss of spoligotype spacers could be used in the same way as the loss of RDs to identify clades within the MTBC. Thanks to the unidirectional evolution of the DR region (Groenen *et al.*, 1993; Fang *et al.*, 1998; van Embden *et al.*, 2000), spoligotypes can be exploited in a similar way than the RDs, although only to a limited extent. This recently proposed scenario is consistent with previous ones (Brosch *et al.*, 2002; Mostowy *et al.*, 2002), but includes the supposed ancestor of each lineage based on the absence or presence of spoligotype spacers (Figure 7). Smith and colleagues (2006b) were also able to predict the spoligotype pattern for each clade in the RD9 deleted lineage of the MTBC. Thus, all RD9 deleted strains lack spacers 9 and 39, “ancestor 4” and its descendants additionally lack spacer 16 and “ancestor 5” and its descendants also lack spacers 3 and 40 to 43. Moreover, Smith and colleagues (2006a; 2006b) incorporated information on the host preference, suggesting

the concept of ecotypes for the MTBC. According to Cohan (2002), selective sweeps might be the cause for limited divergence within groups of bacteria and these groups should be named ecotypes. Through adaptation of a strain to a new niche it becomes immune to periodic selection events (selective sweeps) in the other clades and therefore has the characteristics of an ecotype.



Host	Human	Human?	Human?	Antelope	Seal	Vole	Goat	Cattle
Species	*M. tuberculosis*	*M. africanum* West African I	*M. africanum* West African II	*M. bovis* (antelope) "Oryx bacillus"	*M. pinnipedii*	*M. microti*	*M. caprae*	*M. bovis*

Ancestral spoligotype pattern	
anc 1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■
anc 2	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■
anc 3	□□□■■■■□□□□□□□□□□□□□□□■■■■□□■■■■■■■■■■□□□□□
anc 4	□□□■■■■□□□□□□□□□□□□□□□■■■■■■■■■■■■■■■□□□□□
anc 5	□■□□□□□□□□□□□□□□□■■■■■■■■■■■□■■■■■■■■■□□□□□
anc 6	■■□■■■■■□■■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□

Figure 7. The phylogenetically informative mutations in the lineage leading to *Mycobacterium bovis*. The members of the *Mycobacterium tuberculosis* complex (apart from *M. canettii*) are shown as a series of clades, containing host-adapted ecotypes (Smith *et al.*, 2006a), distinguished by phylogenetically informative mutations. Coloured circles, marked anc1–anc6, represent single-cell ancestors. The distribution of phylogenetically informative deletions (loss of spacers from the DR and deletions of RDs) and phylogenetically informative single nucleotide mutations are shown (Brosch *et al.*, 2002; Mostowy *et al.*, 2002). Species and preferred host are shown. The host for the two clades of *M. africanum* is not fully resolved and is therefore labelled with a question mark. The sequential loss of spoligotype spacers in this lineage is shown. The spoligotype pattern in the single cells represented by anc1–anc6 is shown as a series of 43 boxes (black box indicates presence of the spacer). Adapted from Smith *et al.* (2006b) with permission from the author.

The suitability of a deletion as a phylogenetic marker depends on the stability

and essentiality of the affected genomic region; deletions in regions with a high mutation rate, such as regions with nonessential genes or with repetitive DNA may be prone to deletion and therefore do not offer ideal markers. For this reason, the use of spoligotyping and VNTR typing is limited in terms of phylogenetic studies. Nevertheless, associations between spoligotype patterns and certain lineages have previously been observed for the MTBC (Brosch *et al.*, 2002; Mostowy *et al.*, 2002; Hirsh *et al.*, 2004; Mostowy *et al.*, 2005; Smith *et al.*, 2006a; Flores *et al.*, 2007; Kato-Maeda *et al.*, 2011). Certain features of spoligotype patterns, called spoligotype signatures (Streicher *et al.*, 2007), have been recently used in combination with specific chromosomal deletions to further delineate the global phylogeny of *M. bovis* (Müller *et al.*, 2009; Berg *et al.*, 2011; Smith *et al.*, 2011).

Müller and colleagues (2009) described a geographically localised group of strains of *M. bovis* sharing a distinct spoligotype signature, absence of spacer 30, and a 5.3 kb deletion named RDAf1. The deletion had initially been identified by DNA microarray analysis and a four primer PCR was designed for further large-scale testing. The RDAf1 deletion affects *Mb0587c* to *Mb0589c* and parts of *Mb0586c* and *Mb0590c* (corresponding to *Rv0572c* to *Rv0574c* and parts of Rv0571c and Rv0575c in *M. tuberculosis* H37Rv). The group of related *M. bovis* strains was designated the African 1 (Af1) clonal complex and it was found at high frequency in Mali, Cameroon, Nigeria and Chad, but was not common in other areas of Africa. The authors suggested that the most recent common ancestor of the Af1 clonal complex would have had spoligotype pattern SB0944 (11011111011111101111111111111101111111100000) and also RDAf1 deleted since all Af1 strains can be derived from this pattern by loss of spacers. They also propose that the simplest explanation for the distribution of the Af1 clonal complex is that a single strain or clonal complex entered the four countries and subsequently spread in cattle naïve to bovine tuberculosis.

A different clonal complex of *M. bovis* in East Africa was described by Berg and colleagues (2011), the African 2 (Af2) clonal complex. The distinct spoligotype signature of strains of the Af1 clonal complex was defined as the absence of spoligotype spacers 3 to 7. A specific 14.1 kb deletion, identified by DNA microarray, affected twelve ORFs: *Mb0600c* to *Mb0609c* and parts of *Mb0599c* and *Mb0610c* (corresponding to *Rv0585c* to *Rv0593* and parts of *Rv0584c* and *Rv0594c* in *M. tuberculosis* H37Rv). This deletion was named RDAf2 and interestingly comprised the whole *mce2* operon. Previous reports have described attenuation of *mce2*-deleted isolates of M. *tuberculosis* isolates in the mouse model (Gioffre *et al.*, 2005; Aguilar *et al.*, 2006; Marjanovic *et al.*, 2010). Further studies are needed to determine whether this is also the case for *mce2*-deleted *M. bovis* strains; yet they are known to produce tuberculosis-like lesions in cattle. The progenitor of the Af2 clonal complex would have been a strain with

spoligotype pattern SB0133 (110000010111111011111111111111111111100000) and also the RDAf2 deletion. The distribution of the Af1 and Af2 clonal complexes is shown in Figure 8.

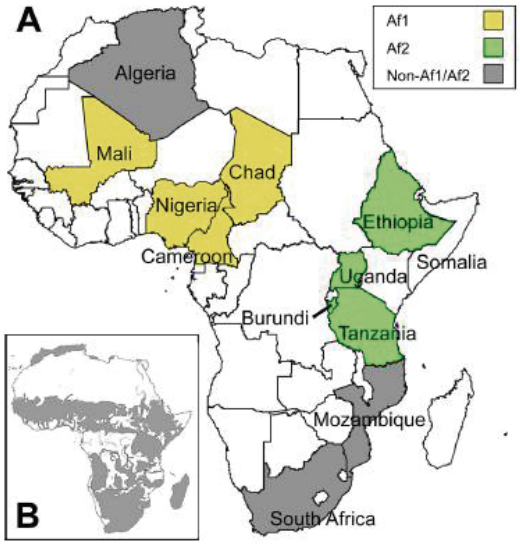


Figure 8. Localisation of the *Mycobacterium bovis* Af1 and Af2 clonal complexes in Africa. (A) The African countries where Af1 was found at high frequency are shown in yellow, and East African countries where Af2 was highly prevalent are shown in green. Countries where neither Af1 nor Af2 strains were identified are labelled in grey. (B) Cattle distribution on the African continent (grey shaded areas). Adapted from Berg *et al.* (2011) and Hanotte et al (2002) with permission from Infection, Genetics and Evolution © and Science ©.

The first clonal complex of *M. bovis* described in Europe, European 1 (Eu1), was found to be at virtual fixation in the UK and the Republic of Ireland (RoI) (Smith *et al.*, 2011). Most of the strains in the British Isles lack spacer 11 which was designated as the spoligotype signature for the Eu1 clonal complex. This clonal complex was also marked by a phylogenetically informative deletion of 806 bp located within gene *treY* (*Mb1589c*, corresponding to *Rv1563c* in *M. tuberculosis* H37Rv). Representative sets of strains were deletion typed for RDEu1 and it was shown that this clonal complex was globally important. The simplest explanation for the wide distribution of members of the Eu1 clonal complex is the large amount of UK trading partners and English speaking former colonies of the UK (Cataldi *et al.*, 2000). Moreover, the authors point out that the UK exported Hereford beef cattle during the 18th century which would have provided an excellent vehicle for distributing this clonal complex.

It is important to underline that spoligotype signatures are a useful tool to hint at membership of a certain clonal complex, but due to the occurrence of homoplasies in the DR region (identical spacer loss in unrelated lineages) are not suitable phylogenetic markers on their own (Smith *et al.*, 2011).

At present, it is thought to be unlikely that the first strain which caused bovine tuberculosis derived directly from *M. tuberculosis*. It is more likely that a RD9 deleted strain, such as *M. africanum* (Figure 7) which is able to be maintained in the human population, gave rise to the animal adapted lineages. Considering that the main geographical localisation of strains of *M. africanum* is the African continent (see section 2.3), the animal lineages might have evolved there (Smith *et al.*, 2009a).

3.4. Spread of the *M. tuberculosis* complex

Modern genetic and archaeological evidence suggests that the domestication of European cattle took place in the Near East at the beginning of the Neolithic (Beja-Pereira *et al.*, 2006; Edwards *et al.*, 2007). Those studies are based on mitochondrial DNA of cattle origin and allow a hypothesis on the expansion of cattle from an ancestral population in the Fertile Crescent (including today's Iraq, Syria, Jordan, Israel, Lebanon, the West Bank and parts of Egypt, Iran and Turkey) (Götherström *et al.*, 2005; Beja-Pereira *et al.*, 2006). Goats were domesticated around the same time in this region and were together with cattle one of the first domesticated animals; evidence of goat domestication was also found in the Indus Valley, China and Mesoamerica (Boyazoglu and Hatziminaoglou, 2004; Boyazoglu *et al.*, 2005).

The introduction of cattle into Europe occurred along with human migration following land routes, but also by maritime routes which is reflected in the strong influence of cattle of North African origin in Mediterranean countries (Figure 9). The same scenario is probably valid for the goat population, since cattle as well as goats are clearly associated with mankind.

Infected cattle and goat populations probably gave rise to locally adapted strains. This evolutionary scenario is congruent with the demography of the MTBC and its association with the human host (Wirth *et al.*, 2008; Hershberg *et al.*, 2008). Nevertheless, it is unknown if bovine and caprine tuberculosis was introduced along with the first livestock arriving into Europe or maybe later on infecting cattle and goats that were naïve to the disease. The pathogen would then have taken advantage of an ecological niche which led to clonal expansion of the founder strains. This is coherent with the hypothesis of ancient cattle being infected with ancestral *M. bovis* strains which present a maximum number of spacers in the DR region. This ancestor would have derived from a *M. tuberculosis*-like organisms (Brosch *et al.*, 2002; Mostowy *et al.*, 2005) and would have generated strains that became adapted to different hosts [cattle (*M. bovis*) and goats (*M. caprae*)] (Smith *et al.*, 2006b). The MRCA of all *M. bovis* and *M. caprae* strains had spacers 3, 9, 16 and 39-43 missing (Smith *et al.*, 2006a).

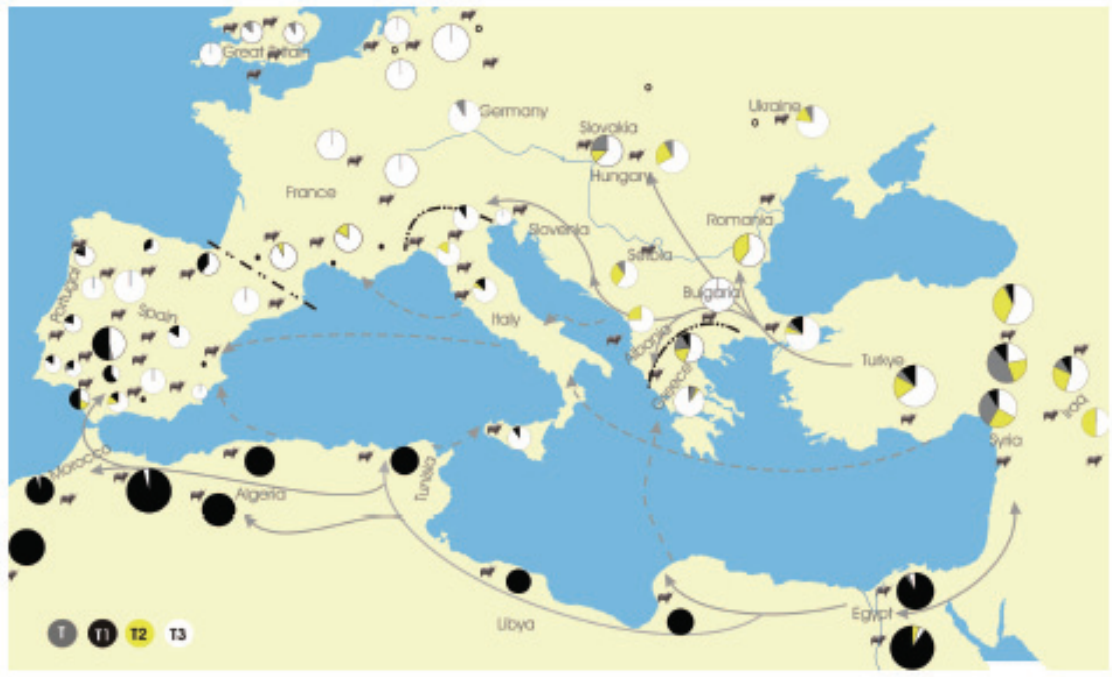


Figure 9. European spread of agropastoralism. Each black cattle figure represents a population sample point. Different hypothesized maritime routes (dashed line with arrow) and continental route (solid line with arrow) are indicated. Dash-dot lines are suggestive of the geographic limits of African cattle influence in Europe. Pie chart represents the frequencies of the four major mtDNA haplogroups, with circle sizes proportional to sample sizes. From Beja-Pereira *et al.*, 2006 with permission from PNAS © (2006) National Academy of Sciences, U.S.A.

Although recent phylogenetic studies of chromosomal deletions and SNPs have contributed to a more detailed vision of the evolutionary scenario for the MTBC and also for *M. bovis* in particular (Brosch *et al.*, 2002; Smith *et al.*, 2006b; Müller *et al.*, 2009; Berg *et al.*, 2011; Smith *et al.*, 2011), the theories on its age and spread remain speculative. Further whole genome analysis of a variety of strains of different origin will be needed in order to establish well-founded phylogenies. Several sequencing projects are ongoing and hopefully will contribute to a more refined understanding of the evolution of the MTBC and in particular *M. bovis* and *M. caprae*.

4. Tuberculosis in animals

M. bovis is considered the main causative agent of animal tuberculosis and from the beginning, the term bovine tuberculosis focused on tuberculosis caused by this pathogen in cattle. Yet, according to the Task Force Bovine Tuberculosis Subgroup (2006) bovine tuberculosis is "Infection in cattle with any of the disease-causing mycobacterial species within the *M. tuberculosis* complex". Although this definition only aims at cattle, the important role of other animal species and the elevation of some of the MTBC members to species rank, *M. caprae* (Aranaz *et al.*, 2003) and *M. pinnipedii*

(Cousins *et al.*, 2003), led to generalisation of the term bovine tuberculosis. Some authors also misleadingly define disease in humans caused by *M. bovis* as bovine tuberculosis. Bovine tuberculosis is a listed disease of the OIE (Office Internationale des Epizooties; http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2011/). *M. caprae* is regarded as a common cause of bovine tuberculosis (Prodinger *et al.*, 2005; OIE, 2009), although it was initially defined as the aetiological agent of infection in goats, so-called caprine tuberculosis (Aranaz *et al.*, 1999; Aranaz *et al.*, 2003).

4.1. Relevance of bovine tuberculosis

Emil von Behring stated in his Nobel lecture of 1901 "As you know, tuberculosis in cattle is one of the most damaging infectious diseases to affect agriculture. It causes premature death in affected animals, damages nutrition and milk production and is the cause of inferior meat." (http://nobelprize.org/nobel_prizes/medicine/laureates/1901/behring-lecture.html). This remains true until today with livestock being infected in 70% of the 178 OIE Member Countries (World Organisation for Animal Health, www.oie.int, consulted on 24 August 2011) and with the decrease in milk and meat production and in fertility being the most important losses due to bovine tuberculosis. A decrease of 10-20% was estimated for the milk and meat production (Bennett and Cooke, 2006; Boland *et al.*, 2010) conditioned by the legal restrictions (Council Directive 64/432/EEC) for sale and/or export of meat and milk from infected animals. In 1995 it was estimated that more than 50 million cattle were infected causing an annual economic loss of over 2 billion Euros (Steele, 1995). The livestock sector occupies nowadays a quarter of emerged land on the globe, including a third of arable land; data that reflect its inexorable growth and importance (Gerber *et al.*, 2010). This is due to the increasing demand for meat, eggs and other animal products following demographic growth, urbanization and economic development (Steinfeld *et al.*, 2006). This situation requires stable and well-defined policies in order to control and improve livestock management including livestock diseases such as tuberculosis.

Globally, bovine tuberculosis has a wide distribution, but with significant differences in prevalence between the countries. In most industrialised countries the disease has been eradicated or reduced drastically thanks to eradication policies. In contrast, in developing countries the disease is still considered endemic. The overall prevalence of bovine tuberculosis is less than 0.001% in the USA and Canada (United States Department of Agriculture, 2009; Canadian Food Inspection Agency, 2010), and in the European Union many countries have reached levels below 0.1% (EFSA, 2011). In Central America, except from Nicaragua and the Caribbean, the prevalence of infected animals is less than 1% (Abalos and Retamal, 2004; de Kantor and Ritacco, 2006), while Cuba is considered free from bovine tuberculosis (Abdala, 1998). The highest

prevalences are found in South America (de Kantor and Ritacco, 2006) and in Africa (Cosivi *et al.*, 1998). Especially in Ethiopia the situation is worrying with prevalence rates ranging from 3.4% in small farms to 50% in intensive productions (Shitaye *et al.*, 2007). The prevalence rates in Asia also vary strongly with prevalences of at least 1%. Only seven nations benefit from a systematic eradication programme, including Iran, where bovine tuberculosis was successfully reduced to an incidence of 0.5% (Mosavari *et al.*, 2011).

Within the European Union, the bovine tuberculosis situation differs strongly between the different countries. According to the European Food Safety Authority (EFSA, 2011) 13 Member States (MSs) (Austria, Belgium, The Czech Republic, Denmark, Germany, Finland, France, Luxembourg, The Netherlands, Poland, Slovakia, Slovenia and Sweden), two non-MSs (Switzerland and Norway), as well as Scotland (UK) and four regions and 20 provinces in Italy, are officially bovine tuberculosis-free (OTF) (Figure 10). The highest herd prevalence of bovine tuberculosis is found in the RoI (5.97% in 2008) and the UK (5.41% in Great Britain and 6.12% in Northern Ireland in 2009). In OTF countries and non-OTF countries a slightly increasing trend has been observed in the last few years (EFSA, 2011; Schiller *et al.*, 2011). Yet, a statistically significant, decreasing trend was observed from 2004 to 2009 in the prevalence of cattle herds tested positive for tuberculosis in the EU cofinanced MSs Italy, Portugal and Spain (EFSA, 2011).

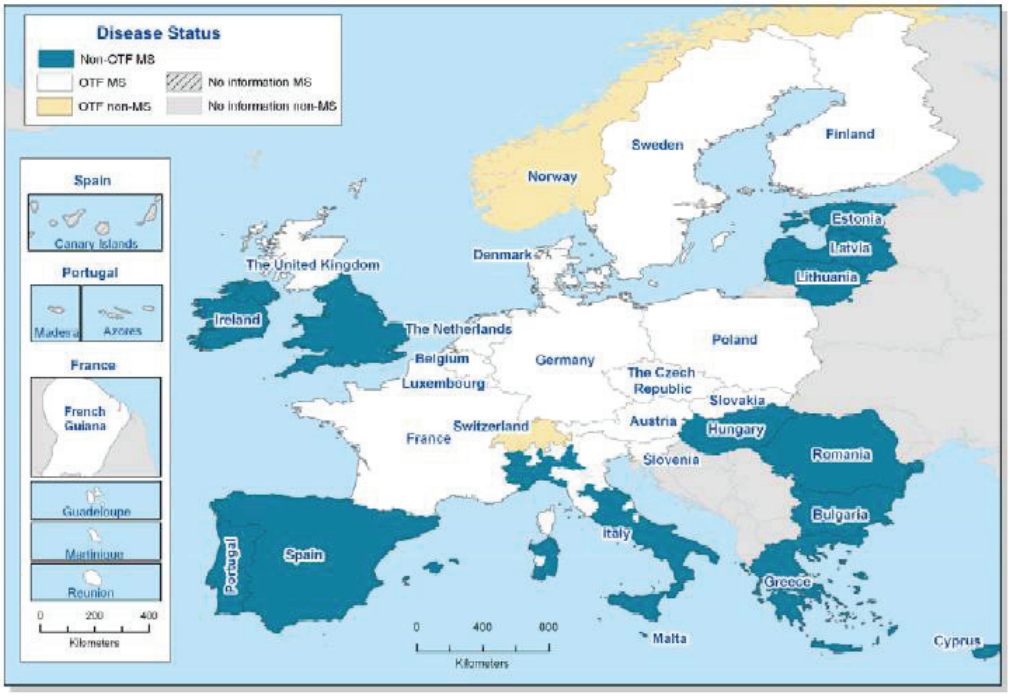


Figure 10. Status of bovine tuberculosis, 2009. European Food Safety Authority (2011).

In Spain, attempts to eradicate bovine tuberculosis started in 1950 in dairy cattle in the Northern regions of the country, but it was not until 1986 that all Spanish regions were subjected to a national eradication programme. Since then, due to the test-and-slaughter policy and systematic sampling of tuberculous lesions at routine abattoir inspections, Spain achieved a herd prevalence rate of 1.51% in 2010 (MARM, Ministerio de Medio Ambiente, y Medio Rural y Marino) (Figure 11).

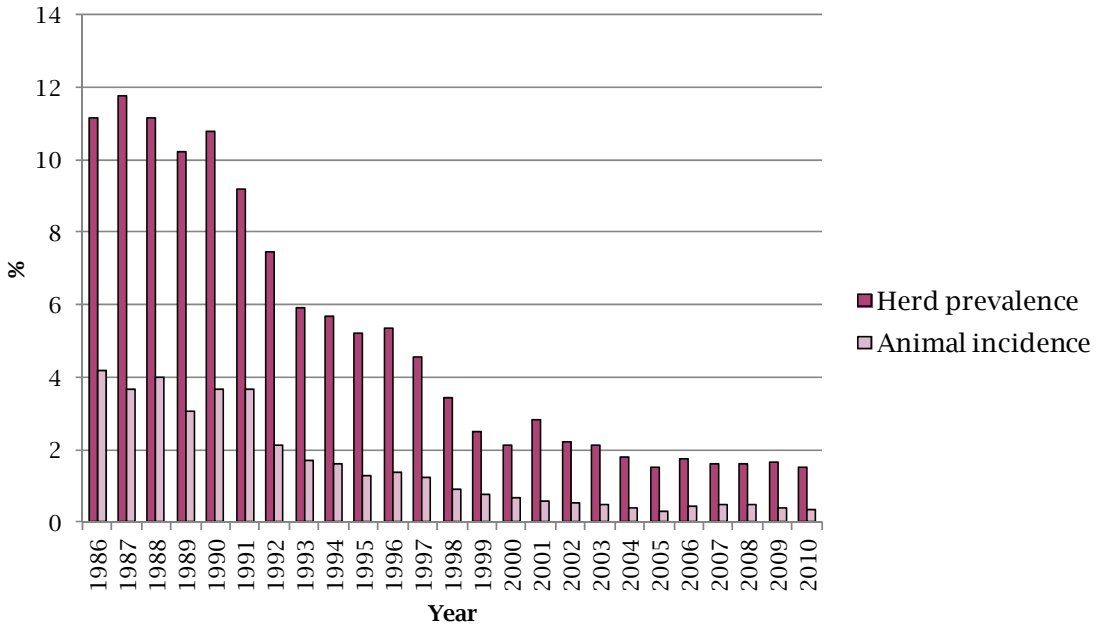


Figure 11. Herd prevalence (purple) and animal incidence (pink) of bovine tuberculosis in Spain, 1986-2010 (MARM, 2010).

Large differences exist between geographical areas with higher rates in the South and Centre of the country (Castilla-La Mancha, Andalucía, Madrid, Extremadura and Murcia) and lower incidence levels in Northern Autonomous Communities (Asturias, Galicia and Navarra) (Table 3). Cattle in the Balearic and Canary Islands are considered free from tuberculosis since 2008 and 2009, respectively. The geographical distribution of bovine tuberculosis in Spain could be due to i) the climatic conditions, ii) the production system and iii) the presence of wildlife. In the semiarid regions of the south and centre of the country artificial watering sites promote the aggregation of animals which favours the spread of the disease. A recent study confirmed that these regions are at higher risk for maintaining bovine tuberculosis (Allepuz *et al.*, 2011). The production system is another factor to be taken into account, because extensive management of beef cattle, including common but also autochthonous cattle breeds which are more difficult to handle, is more often found in South and Central Spain. In extensive management the animals habitually share pasture with animals from different farms and also with other animal species, for example goats. Dairy cattle can be mainly found

in the North and it should be underlined that the eradication programme was first implemented in dairy cattle so that eradication of bovine tuberculosis in this production system is in an advanced stage. Wildlife is known to act as a reservoir host and favours the maintenance of the disease. In Spain the most important species involved in bovine tuberculosis are Eurasian wild boar and red deer (Aranaz *et al.*, 2004a; Gortázar *et al.*, 2005; Parra *et al.*, 2006; Martín-Hernando *et al.*, 2007; Naranjo *et al.*, 2008; Martín-Hernando *et al.*, 2010; Gortázar *et al.*, 2011b) which show highest population densities in South-central Spain (Vicente *et al.*, 2007). The combination of these factors might slow down the eradication of bovine tuberculosis in Southern and Central Spain.

Table 3. Herd prevalence of bovine tuberculosis in the Autonomous Communities of Spain from 2005-2010 (Ministerio de Medio Ambiente Rural y Marino, MARM).

Autonomous Community	2005	2006	2007	2008	2009	2010
Andalucía	5.32	5.76	4.15	5.80	8.94	8.54
Aragón	1.56	1.96	3.65	0.75	0.70	1.22
Asturias	0.18	0.17	0.24	0.22	0.21	0.18
Baleares	0.65	0.22	0.21	0.00	0.00	0.17
Canarias	1.00	0.36	0.37	0.24	0.00	0.00
Cantabria	1.16	1.05	2.25	1.57	0.91	0.79
Castilla-La Mancha	7.02	7.71	9.51	11.62	10.27	7.11
Castilla y León	3.37	5.11	4.16	3.71	2.75	2.62
Cataluña	1.70	1.65	1.08	0.85	0.83	0.59
Extremadura	4.05	4.84	3.74	3.37	3.78	3.04
Galicia	0.31	0.20	0.19	0.11	0.22	0.28
La Rioja	1.31	0.72	0.70	1.45	0.75	1.14
Madrid	2.58	2.59	3.41	5.72	5.54	5.45
Murcia	4.46	4.96	8.05	3.29	3.51	1.59
Navarra	0.38	0.27	0.33	0.40	0.30	0.67
País Vasco	0.64	0.19	0.14	0.20	0.57	0.37
Valencia	2.16	1.61	1.14	1.41	1.38	3.84
TOTAL	**1.52**	**1.76**	**1.63**	**1.59**	**1.65**	**1.51**

4.2. Relevance of caprine tuberculosis

Although the goat milk industry is small compared to the bovine industry in the developed world, the sector is growing and becoming economically more important (Dubeuf and Boyazoglu, 2008) and tuberculosis has to be considered a serious threat to goat flocks (Shuralev *et al.*, 2011). The first description of infection in this animal species identifies a young goat reared from cow's milk and dates from 1917 (Griffith, 1917). Tuberculosis in goats was recognised early in the U.S.A., Britain and Germany although at low levels (Bishop *et al.*, 1936; Begley, 1938; Soliman *et al.*, 1953, Francis, 1958). Caprine tuberculosis was long thought to be negligible in the UK and the RoI (O'Reilly and Daborn, 1995), but in recent years the number of publications reporting tuberculosis in goats has risen indicating a growing importance (Crawshaw *et al.*, 2008; Daniel *et al.*, 2009; Sharpe *et al.*, 2010). Infection was caused by *M. bovis*, but no cattle contact could be confirmed, although one report mentions a possible contact to sheep

or feral goats (Daniel *et al.*, 2009). In Europe, goats infected with *M. bovis* have also been found in France (Haddad *et al.*, 2001) and Portugal (Duarte *et al.*, 2008; Quintos *et al.*, 2010; Cunha *et al.*, 2011b). Reports from Nigeria and Ethiopia confirm the presence of *M. bovis* and *M. tuberculosis* in goats (Cadmus *et al.*, 2009; Hiko and Agga, 2011), and a recent study from Pakistan reports skin-test-positive animals in all of the farms studied (Javed *et al.*, 2010). Caprine tuberculosis is also present on the American continent according to a recent study in Brasil (Marassi *et al.*, 2009) and was also described in Australia (Cousins *et al.*, 1993) and New Zealand (Sanson, 1988) in earlier years.

Caprine tuberculosis in Spain is mainly caused by *M. caprae* (Aranaz *et al.*, 1999). This pathogen is known to affect other animal species and humans and has also been recognised in cattle and other domesticated and non-domesticated animal species in continental Europe. Many central European countries, where *M. bovis* is generally not found, have reported *M. caprae* isolations (Pavlik *et al.*, 2002a; Pavlik *et al.*, 2002b; Erler *et al.*, 2004; Prodinger *et al.*, 2005; Pate *et al.*, 2006; Csivincsik *et al.*, 2008). Other reportedly affected European countries are France (Haddad *et al.*, 2001), Greece (Ikonomopoulus *et al.*, 2006), Portugal (Duarte *et al.*, 2008) and Italy (Boniotti *et al.*, 2009). The prevalence of *M. caprae* infections is difficult to assess, on the one hand because it is has long been (and sometimes still is) referred to as *M. bovis*, and also because caprine tuberculosis is generally not included in eradication programmes.

Tuberculosis in goats is of special concern in countries with an important goat production. According to the Food and Agriculture Organization of the United Nations (FAO) only 4.2% of the worldwide goat population is located in the developed world, while the developing countries account for 95.8%. In Europe Greece, Spain and France are leading in the goat industry (Figure 12).

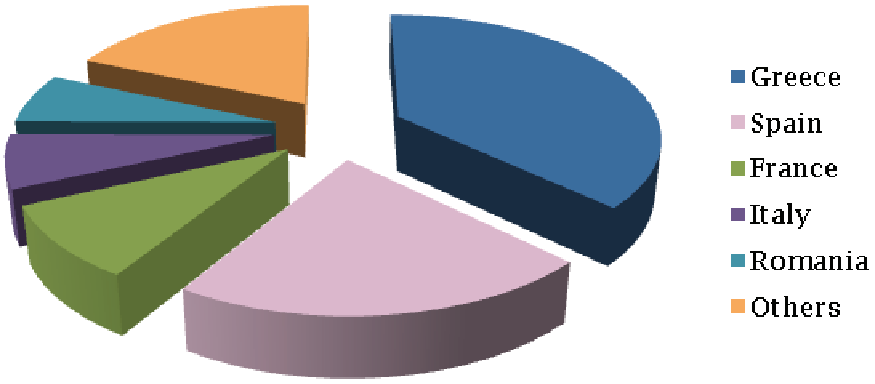


Figure 12. Distribution of the goat population in the European Union in 2007. Source: Eurostat (2008).

Caprine tuberculosis is not included in the list of diseases notifiable to the OIE and therefore not subjected to eradication campaigns. Nevertheless, it is advisable to monitor tuberculosis-infected goat herds and goats sharing farms with cattle, because of the high economic impact due to decreased goat production, increased mortality rates and costs of diagnosis (Bezos *et al.*, 2011). In Spain, the current eradication programme includes small ruminants that co-exist with cattle in the same farm, and several Autonomous Communities have started local eradication programmes in goats (Murcia, Castilla y León, the Canary Islands and Andalucía). Losses due to *M. caprae* are compensated to the farmers in cattle and goats that co-exist with cattle (Royal Decree 389/2011). However, there is only scattered information on the prevalence of the disease in goats; most of the small-scale studies available to date reported an animal prevalence of over 15% and a herd prevalence of over 80% (García Marin, 2010). For example, a study of goat flocks in the Autonomous Community of Asturias by Balseiro and colleagues (2001) found 92% of the flocks to be infected.

The emergence of *M. caprae*, especially in countries that are not officially free from bovine tuberculosis, suggests that it might be necessary to address *M. caprae* infections when an important goat population is present.

4.3. Tuberculosis in other animal species

Tuberculosis caused by *M. bovis* and *M. caprae* can affect a wide range of animal species. Isolations of *M. bovis* have been made from buffaloes, bison, sheep, goats, equines, camels, pigs, wild boar, deer, antelopes, dogs, cats, foxes, minks, badgers, ferrets, rats, primates, alpacas, llamas, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (de Lisle *et al.*, 2001; O'Reilly and Daborn, 1995). While infections in domesticated animals other than cattle and goats are less frequent, the presence of the pathogens in wildlife is cause for concern since they can act as a reservoir of the infection. Furthermore, tuberculosis threatens endangered species, such as the Iberian lynx (Briones *et al.*, 2000; Peña *et al.*, 2006; Gortázar *et al.*, 2008), lions or cheetahs (*Acinonyx jubatus*) (OIE, 2009) by increasing the morbidity and mortality of these animal hosts (Daszak *et al.*, 2000). Lastly, an impact on public health is possible by exposure of hunters/veterinarians to animal carcasses and through the consumption of products from game animals.

Wildlife plays an important role in the maintenance and transmission of *M. bovis* and therefore hampers the control of the disease in Europe (Wilson *et al.*, 2009). A well-studied wildlife reservoir is the badger (*Meles meles*) population in Great Britain and Ireland; *M. bovis* infection has been found to have a minor impact on mortality and

fertility in this animal species (Nolan and Wilesmith, 1994; Eves, 1999; Gallagher and Clifton-Hadley, 2000; Delahay *et al.*, 2002; Gormley and Costello, 2003). In Britain and Ireland, badgers live at relatively high density and often make contact with livestock at pasture and in farm buildings. Prevalence in badgers ranged from 2% to 37% in a study from 2007 in the South and West of Britain, where the highest prevalence of bovine tuberculosis is found (Bourne *et al.*, 2007). A study conducted in four areas in Ireland revealed a prevalence of *M. bovis* of 19.5% in badgers (Griffin *et al.*, 2005). Wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) are also highly susceptible to infection and high prevalence of tuberculosis can be reached, in particular in parts of the Iberian Peninsula, where wild boar and red deer are maintenance hosts (Aranaz *et al.*, 2004; Hermoso *et al.*, 2006; Naranjo *et al.*, 2008). The highest prevalence observed to date in wild ungulates in Spain occurs in the South and Centre of the country with local prevalence of up to 52% in wild boar and 27% in red deer (Vicente *et al.*, 2006; Gortázar *et al.*, 2008). Infection in wild boar is widespread in continental Europe and has been found in OTF and non-OTF countries; several reports confirmed the disease in Hungary (Machackova *et al.*, 2003; Erler *et al.*, 2004), France (Haddad *et al.*, 2001; Zanella *et al.*, 2008), Germany (Schultz *et al.*, 1992), Italy (Serraino *et al.*, 1999; Boniotti *et al.*, 2009), Portugal (Duarte *et al.*, 2008; Santos *et al.*, 2009) and in the Czech Republic and Slovakia (Pavlik *et al.*, 2002a; Machackova *et al.*, 2003). In Europe tuberculosis in cervids has also been reported from the UK (Delahay *et al.*, 2007), Ireland (Quigley *et al.*, 1997), Austria (Prodinger *et al.*, 2002; Glawischnig *et al.*, 2003), Switzerland (Bouvier, 1963), Hungary (Pavlik, 2006), Portugal (Duarte *et al.*, 2008), France (Zanella *et al.*, 2008) and Denmark (Clausen and Korsholm, 1991). The prevalence varies between different countries and regions and is estimated to range from 1% to 27% (Wilson *et al.*, 2009).

The involvement of wildlife in bovine tuberculosis has also been reported overseas in feral pigs and Asian water buffalo (*Bubalus bubalis*) in Australia (McInerney *et al.*, 1995; Cousins *et al.*, 1998b), Brushtail possums (*Trichosurus vulpecula*) in New Zealand (Tweddle and Livingstone, 1994; Coleman *et al.*, 1994; Ryan *et al.*, 2006), African buffalo (*Syncerus caffer*) in South Africa (Bengis *et al.*, 1996; Michel *et al.*, 2006), whitetail deer (*Odocoileus virginianus*) in Michigan, USA (Schmitt *et al.*, 2002), bison (*Bison bison athabascae*) in Canada (Nishi *et al.*, 2003; Wobeser, 2009), and feral swine (*Sus scrofa*) on Molokai Island, Hawaii (Essey *et al.*, 1983; Bany and Freier, 2000).

An infected wild animal population can be classified as either maintenance or spillover host, depending on the dynamics of the infection (Morris and Pfeiffer, 1995). In a maintenance host, infection can persist by intra-species transmission, and is also a source of infection for other species. In a spillover host, infection cannot persist unless re-infection from another species occurs. Although a spillover host is mainly a dead end (or end stage) host, it may also represent a risk of infection for livestock through

spillback (Corner, 2006; Palmer, 2007). Under these terms, badgers in the British Isles, wild boar and red deer in Spain and possums in New Zealand can be classified as maintenance hosts. In contrast, feral pigs and Asian water buffalo in Australia which did not play a significant role for tuberculosis in cattle, were considered a spillover and dead end host. In Spain, spillover hosts include red fox (Millán *et al.*, 2008), roe deer (Balseiro *et al.*, 2009) and Iberian lynx (Briones *et al.*, 2000; Peña *et al.*, 2006). The role of badgers in Atlantic Spain remains undefined since infection seemed negligible until recently, but an increase of the badger population has been observed during the last years (Sobrino *et al.*, 2009; Balseiro *et al.*, 2011).

Interestingly, the Balearic and Canary Islands, where cattle is bovine tuberculosis free, lack all four potential wildlife reservoirs, while the Italian Mediterranean island Sicily, where tuberculosis has been diagnosed in feral pigs, continues to struggle with tuberculosis in livestock (Boniotti, 2010). A similar situation to Sicily was described for Corsica, where domestic and wild animal species live in close contact (Richomme *et al.*, 2010).

For the control of tuberculosis in wildlife it is essential to not only report the outbreaks, but to give management recommendations at the same time. A new approach to understanding the epidemiology in wildlife involves links between wildlife pathogens, the environment and human activity; this new research field is called “disease ecology” (Gortázar *et al.*, 2007). To further improve current control policies a better understanding of risk factors in relation to tuberculosis infection is indispensable (Vicente *et al.*, 2007). Studies have to be conducted on waterhole ecology, wildlife space use and movements, carrion and gutpile consumption by birds and mammals and the effect of attempts to control tuberculosis in wildlife, e. g. through population control or vaccination (Gortázar *et al.*, 2011b). Furthermore, new serological tests are being investigated which will improve disease surveillance and management of wildlife, for example wild boar (Boadella *et al.*, 2011).

4.4. Legal framework and economics

The importance of controlling bovine tuberculosis has been recognised early and has been a major objective of farming communities and public authorities for almost a century. First initiatives in the European Union (EU) aimed at facilitating intra-community trade by establishing general animal health requirements. The main pillar of today’s legislation is Council Directive 64/432/EEC of June 1964 which laid down specific requirements for the trade in relation with bovine tuberculosis and defined the “officially tuberculosis-free bovine herd” (TBOF) status. One of the most substantial amendments made since then was Council Directive 97/12/EC of March 1997, which modified the requirements for the TBOF status, and Commission Regulation (EC) No.

1226/2002 of July 2002, which deals with diagnosis and the incorporation of new diagnostic tests. The obligation of MSs to draft and carry out eradication programmes was foreseen in Council Directive 77/3917/EEC of May 1977. Furthermore, the financial framework was defined by Council Decision 90/424/EEC of June 1990. The procedures for the post-mortem inspection at abattoirs are established by Council Directive 64/433/EEC, for example, meat from animals with generalised TB must not be declared fit for human consumption and rules are laid down for the inspection of carcasses of reactor animals. Community measures regarding milk hygiene are given in Council Directive 92/46/EEC which, for instance, laid down that only milk from TBOF herds can be used for human consumption without heat treatment.

A highly relevant component of the control programmes is the ante-mortem diagnosis of tuberculosis which is based on the tuberculin skin test. Infected animals develop a delayed-type hypersensitivity reaction at the injection site (caudal fold or neck) when they are inoculated with the tuberculin, a purified protein derivative (PPD) of *M. bovis* and/or *M. avium*, resulting in swelling of the skin (Schiller *et al.*, 2010). The reading of the test result is due 72 hours after injection and the status of the animal can be negative, positive or inconclusive. The single intradermal cervical tuberculin (SIT) test uses only bovine tuberculin, but cattle can sometimes be infected with other types of mycobacteria which may cause the animal to react to the test. In order to distinguish between animals infected with *M. bovis* and those infected by other mycobacteria, the single intradermal comparative cervical tuberculin (SICT) test can be applied to increase the specificity (Álvarez *et al.*, 2008). In this case, the reactions to bovine and avian tuberculin are compared to determine the test result; this method is recommended in low-prevalence settings and when infection with other mycobacterial species is suspected. Parallel testing with the gamma-interferon (IFN-γ) assay (Bovigam, Prionics, Switzerland), an enzyme-linked immunosorbent assay (ELISA) (Rothel *et al.*, 1990) can be used to increase the sensitivity of the skin test especially in high prevalence herds or regions (Schiller *et al.*, 2010). Recent advances in both antigen discovery and immunoassay technology have facilitated progress in developing novel antibody detection assays which facilitate a cost-effective alternative, for example the multiplex immunoassay (Enfer Scientific) that simultaneously detects and analyzes antibody responses to multiple antigens (Whelan *et al.*, 2008).

Vaccination with the BCG live vaccine may also lead to a positive reaction to the skin test and therefore new vaccines that permit differentiation between vaccinated and infected animals are being developed. To date, no suitable vaccine is on the market and vaccines are not administered as they may compromise the tuberculin skin test or immunological tests to detect infected animals; however, many studies are ongoing (Vordermeier and Hewinson, 2006; Whelan *et al.*, 2011). Wildlife vaccination is also one

of the issues that remain to be addressed within control programmes, since reservoir host species are thought to be responsible for the maintenance of the disease (see section 4.3). Finally, the perspective of a new vaccine is also of great importance to the developing world that cannot afford expensive test-and-slaughter strategies.

Control programmes are primarily focused on prevention, eradication and surveillance. Prevention is meant to reduce the probability of exposure to the pathogen by improving herd hygiene and biosecurity measures, e. g. reducing the contact to neighbouring farms or wildlife. Eradication is based on the test-and-slaughter policy which means removing skin test positive animals, so-called reactors, from the farms. The removal of an entire herd can take place as the most drastic measure within disease eradication programmes, but is considered very efficient provided it is carried out in accordance to well-defined strategies (Task Force Bovine Tuberculosis Subgroup, 2006). The most valuable tool for surveillance is the meat inspection at abattoirs, but also the collection of data concerning the farms.

The legal framework in Spain is laid down in the Royal Decree (*Real Decreto*) 2611/1996, 20 December, amended by the Royal Decree 1074/2003, 18 October, which regulates all national eradication programmes of animal diseases and most importantly specifies the skin test measures and defines the status of tuberculosis infected, suspended or free farms.

Regarding the economics of bovine tuberculosis Torgerson and Torgerson (2008; 2010) suggested that the large sums spend on the eradication of the disease are disproportionate to the benefits and based on out-of-date fears of transmission to humans. Yet, their statement is controversial (Smith and Clifton-Hadley, 2008; Gordon, 2008). It should be taken into account that the economic assessment of the eradication strategies is mainly focused on the expenses of control efforts, for example the premovement testing (Bennet, 2009), but rarely includes the analysis of the benefits, such as elimination of the loss in milk and meat production, stock replacement or premovement testing (Zinsstag *et al.*, 2006). Besides, bovine tuberculosis causes considerable costs on the personal well-being of farm households and also raises livestock welfare issues, which must not be ignored (Butler *et al.*, 2010).

4.5. Pathogenesis

The clinical expression of infection with tuberculosis depends on a number of factors including route of transmission, host features, virulence of the mycobacterial strain and infective dose (Neill *et al.*, 1994). Aerosols are considered the most frequent route of transmission regarding animals (Menzies and Neill, 2000; Pollock and Neill, 2002). However, ingestion of contaminated material, feed and milk, is also important,

above all when the climatic conditions favour the survival of the pathogen on the pastures (Goodchild and Clifton-Hadley, 2001). Oral infection can lead to intestinal lesions, which are rarely found in cows in countries with eradication programmes. Nevertheless, this is an important route of transmission for wildlife, since many wild animals appear to become infected by scavenging infected carcasses (Wilson *et al.*, 2009; Gortázar *et al.*, 2011b). Cutaneous infection, for example caused by bite wounds, is even less frequent. This form of infection is usually limited to the affected region and the corresponding regional lymph tissue. Bite wounds were described as a significant route of transmission in high density badger populations (Macdonald *et al.*, 2004).

Several factors regarding the host also influence the pathogenesis of tuberculosis, most importantly the host‘s immune status and also age, behaviour and the production system. Generally, younger animals develop more severe lesions than adult animals (Martín-Hernando *et al.*, 2007). Moreover, dairy cattle are more frequently affected due to larger herd sizes and increased stress through different management, e. g. milked twice a day; they are also longer exposed to a possible risk of infection because they reach an older age than beef cattle (Ramírez-Villaescusa *et al.*, 2010). However, in Spain it is dairy cattle that show lower prevalence rates probably due to the fact that the control programmes have been established first in this sector.

The strain virulence determines the ability of the pathogen to overcome the host defences and it depends on a series of host-pathogen interactions (Collins, 2001). MTBC members possess different virulence factors, including the ones that are associated to the lipids in the cell wall which act as the most significant defensive, offensive or adaptive effectors of virulence (Hotter and Collins, 2011). Most of the virulence factors known at present were discovered by analysis of the attenuated strains of *M. bovis*, *M. bovis* BCG, and *M. tuberculosis*, *M. tuberculosis* H37Rv.

The distribution and severity of the tuberculous lesions furthermore depend on the infective dose that on its part depends on the route of transmission. The predominant distribution of tuberculous lesions in the respiratory tract and associated lymph nodes of infected cattle led researchers early to the assumption that infection occurred via the aerosol route (Francis, 1947), which is since thought to be the key portal of entry (Cassidy, 2006). According to studies in guinea pigs, oral infection requires significantly higher doses than aerosol infection; airborne transmission only requires 1-5 bacilli to cause disease (Neill *et al.*, 1991; Johnson *et al.*, 2007), which means 1000 times less than via the oral route (Collins and Grange, 1983).

It is important to differentiate infected animals and animals with clinical symptoms. If an animal is exposed to any pathogen included in the MTBC, it develops an immune response to the antigens of the bacteria and will respond positively to

subsequent exposure, for example the skin test, but does not necessarily present clinical signs (Pollock and Neill, 2002). It is important to note that mycobacteria are intracellular bacteria that are able to survive in macrophages during long periods of time without causing disease. Animals with clinical symptoms and/or tuberculous lesions shed bacilli and may infect other mammals. The affected organs generally (but not exclusively) depend on the route of transmission: aerosol infection mainly leads to pulmonary lesions while oral or cutaneous infection usually causes extrapulmonary tuberculosis.

On the basis of the natural history of the disease it can be distinguished between primary and postprimary tuberculosis. Primary tuberculosis occurs after first-time exposure to the pathogen. Subsequently, the host immune system reacts by macrophage-rich response to the mycobacteria and the formation of the typical tuberculous granuloma, so-called tubercle, which represent focal expression of granulomatous inflammation that can restrict or prevent mycobacterial growth. The bacteria are also disseminated by the lymphatic circulation to regional lymph nodes. The tubercles that are found at the portal of entry and in the corresponding lymphoid tissue form the so-called primary complex. If the host immune system cannot keep the bacilli contained and under control, they multiply rapidly, enter the bloodstream and spread throughout the body. This stage of dissemination is called postprimary, or secondary, tuberculosis. Postprimary tuberculosis can also results from either reactivation of a latent primary infection or, less commonly, from the repeat infection of a previously sensitized host.

4.6. Sampling and bacterial identification

The gold standard for confirmation of infection with a MTBC member is the direct detection of the bacteria by bacteriological examination which may consist of the demonstration of acid-fast bacilli by microscopic examination which provides presumptive confirmation, and moreover, by isolation of mycobacteria on selective culture media and their subsequent identification by cultural and biochemical tests or DNA techniques, such as PCR.

4.6.1. Bacteriological culture

Specimens for bacteriological culture consist of tissue with visible lesions, such as caseous necrosis in lymph nodes (above all submandibular, retropharyngeal, tracheobronchial, mediastinal, and mesenteric lymph nodes) and altered parenchymatous organs, e. g. lung, liver or spleen, as well as any tissues with gross lesions. In Spain this is laid down in the “Manual of proceedings for the taking and remittance of samples for the culture of mycobacteria” (Centro VISAVET and MARM, 2006) that is based on Directive 64/432/ECC. The OIE (2009) recommends culturing

pooled lymph node samples from the head and thorax when no visible lesions are detected in tuberculin or interferon test positive animals at post-mortem examination.

Direct microscopy is the fastest, cheapest and simplest way for the detection of acid-fast bacteria in tissue samples. For this purpose, a direct smear of a clinical sample can be stained following the Ziehl-Neelson (ZN) technique or with a fluorescent acid-fast stain, e. g. with auramine (Smithwick, 1976). The initial stain for mycobacteria was developed by Robert Koch in 1882 and was subsequently modified by Paul Ehrlich and Franz Ziehl in the same year; finally, one year later the popular ZN stain was perfected by Friedrich Neelson (Cook, 1997). In combination with characteristic macroscopic lesions a presumptive diagnosis of mycobacteriosis can be made, but confirmation by culture is indispensable.

After macroscopic analysis in order to determine the presence and the nature of the lesion, the samples are chopped and homogenised using a stomacher. The next step is decontamination of the samples with detergents; due to the slow growth of the mycobacteria this measure is indispensable. Different detergents can be used for this purpose: hexadecylpyridiniumchloride (Corner and Trajstman, 1988; Corner *et al.*, 1995), oxalic acid (Nassau, 1958; Claxton *et al.*, 1979) and sodium hydroxide (Kent 1985; Corner and Trajstman, 1988) or in combination with sodium laurylsulfate (Tacquet and Tison, 1961; Mankiewicz and Dernuet, 1970; Corner and Nicolacopoulos, 1988). The mixture is shaken for 30 minutes at room temperature, the suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture. The inoculated culture medium is incubated at 37ºC for three months. The most frequently used culture media are: solid egg-based media, such as LJ (Jensen, 1932), Coletsos (Grange *et al.*, 1996), Stonebrink's (Stonebrink, 1958; Rüsch-Gerdes *et al.*, 1985; Goh and Rastogi, 1991; Carbonnelle *et al.*, 1995), agar-based media like Middlebrook 7H10 or 7H11 (Vestal and Kubica, 1966; Hines *et al.*, 2006) or blood based agar medium (Cousins *et al.*, 1989). The media contain glycerol which is required as carbon source for the growth of *M. tuberculosis*, but cannot be used by *M. bovis* (Kanchana *et al.*, 2000; Hines *et al.*, 2006). The *M. bovis* genome sequence revealed a mutation in the gene that encodes the pyruvate kinase (PK), an enzyme which catalyses the final step in glycolysis (phosphoenolpyruvate → pyruvate) (Keating *et al.*, 2005). Therefore, *M. bovis* does not use glucose as source of energy but amino acids or fatty acids. In order to supplement this enzymatic deficiency and to enhance bacterial growth sodium pyruvate is added to the culture media (Keating *et al.*, 2005). Nowadays, several liquid culture systems are on the market which shorten the incubation period to 42 days instead of three months, and increase the sensitivity of the technique. Examples of liquid culture systems are the BACTEC 460 system (Becton Dickinson Inc.) (Williams-Bouyer *et al.*, 2000; LaBombardi,

2002), the MB/BACT (Organon Teknika, Boxtel, Netherlands) and the BACTEC MGIT 960 (Becton Dickinson Inc.) (Hanna *et al.*, 1999; Hines *et al.*, 2006).

4.6.2. Molecular identification

Rapid identification of bacterial isolates to genus and MTBC level can be achieved by PCR targeting genus-specific 16S ribosomal RNA (rRNA) and MTBC-specific proteins, such as MPB70, in bacterial DNA extracted from the previously described culture media. The rRNA sequences are highly conserved among prokaryotes and harbour stable regions as well as variable regions which are characteristic for almost every group of organisms (Woese *et al.*, 1987). The 16S rRNA presents small but conserved sequence polymorphism and therefore constitutes an ideal target for primers for the amplification of genus specific fragments of mycobacteria (Böddinghaus *et al.*, 1990; Rogall *et al.*, 1990; Tortoli, 2003) (see also section 1.1). This method is commonly used with the oligonucleotides MYCGEN-F and MYCGEN-R that amplify a 1,030 bp fragment which is specific for the genus *Mycobacterium* (Böddinghaus *et al.*, 1990; Wilton and Cousins, 1992). Nevertheless, it cannot be used to differentiate the MTBC members at species level (Glennon *et al.*, 1994).

The protein MPB70 was first isolated from *M. bovis* BCG (Nagai *et al.*, 1981) and is one of the best-studied mycobacterial antigens. The MPB70 proteins of the different MTBC members are highly homologous and a fragment of the *mpb70* gene can be used for molecular identification members of the MTBC (Cousins *et al.*, 1991; Cousins *et al.*, 1992). A multiplex PCR targeting 16S rRNA and the *mpb70* gene allows for rapid identification at genus as well as MTBC level (Wilton and Cousins, 1992; Liébana *et al.*, 1996).

Specific identification of isolates of the MTBC can be made using PCR targeting single nucleotide mutations in genes *oxyR*, *katG*, *pncA*, *gyrA*, *mmpl6* and *gyrB* as described in section 2 (Table 2) (Espinosa de los Monteros *et al.*, 1998; Kasai *et al.*, 2000; Niemann *et al.*, 2000b; Cousins *et al.*, 2003; Aranaz *et al.*, 2003; Chimara *et al.*, 2004; Viana-Niero *et al.*, 2004; Goh *et al.*, 2006; Huard *et al.*, 2006). Moreover, the presence and/or absence of RDs can be exploited for rapid species identification as mentioned above (see sections 2 and 3.3) (Brosch *et al.*, 2002; Mostowy *et al.*, 2002; Huard *et al.*, 2003; Bigi *et al.*, 2005; Huard *et al.*, 2006; Smith *et al.*, 2009b).

Additional molecular typing techniques, such as spoligotyping or IS*6110*-based techniques, able to differentiate between species and applied for intra-species strain typing will be described in detail below (see section 5).

4.7. Zoonotic aspects of *M. bovis* and *M. caprae*

Tuberculosis remains one of the biggest threats to human health, especially for the developing countries and for persons infected with the human immunodeficiency virus (HIV). According to the World Health Organisation (WHO) Report on Global Tuberculosis Control (2010), the estimates of the global burden of disease caused by tuberculosis in 2009 are 9.4 million incident cases (Figure 13), 1.3 million deaths among HIV-negative people and 0.38 million deaths among HIV-positive people. Most cases were reported from South-East Asia, Africa and the Western Pacific regions (35%, 30% and 20%, respectively). The African Region accounted for approximately 80% of the cases with HIV and tuberculosis co-infection. With multidrug-resistant (MDR) and extensively drug resistant tuberculosis (XDR TB) being on the rise (by July 2010, 58 countries and territories had reported at least one case of XDR TB) a new concern has emerged. MDR TB is resistant to at least two of the best anti-tuberculosis drugs, isoniazid and rifampicin, which are considered first-line drugs and are used to treat all tuberculosis patients. XDR TB is defined as tuberculosis resistant to isoniazid and rifampin, plus resistant to any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin) (CDC, 2010). Patients suffering from XDR TB are left with much less effective treatment options.

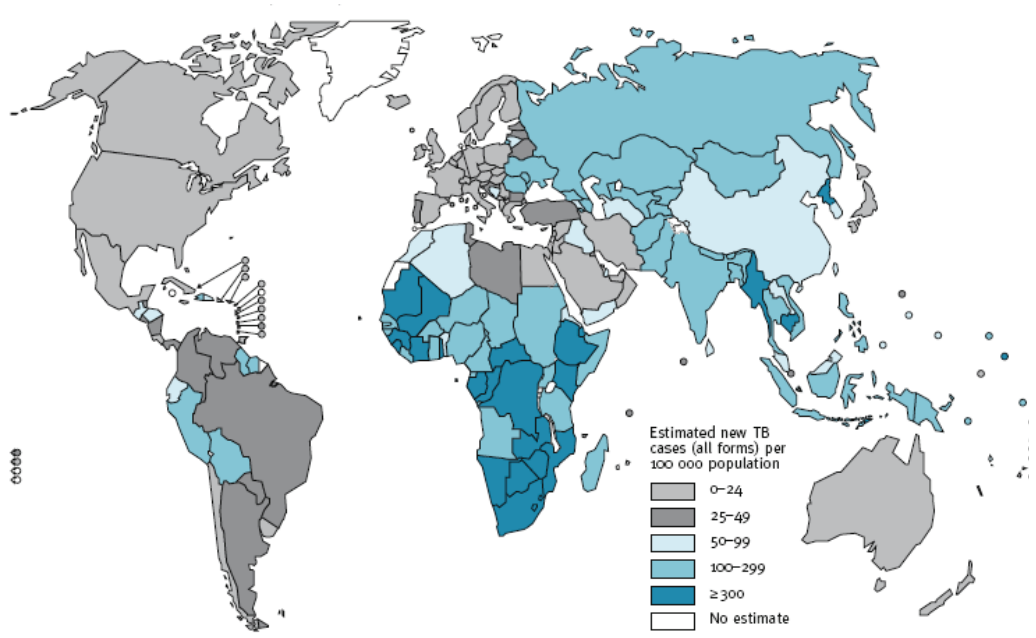


Figure 13. Estimated incidence rates for human tuberculosis by country, 2009. WHO Report on Global Tuberculosis Control (2010).

The major causative agent of human tuberculosis is *M. tuberculosis*. Cases due to *M. bovis* and *M. caprae* have become rare thanks to pasteurisation of milk and dairy products (Grange, 2001; de la Rua-Domenech, 2006). Moreover, it is widely recognised

that the elimination of zoonoses requires its control in the animal reservoir and the eradication programmes for bovine tuberculosis have undoubtedly led to reduction in disease and death caused in the human population. Nevertheless, this disease continues to be important in regions where bovine tuberculosis is poorly controlled and also threatens human beings with a deficient immune system. Furthermore, an occupational risk exists for farmers, abattoir staff or veterinarians who work in close contact with cattle, and wildlife poses a risk to hunters (Fanning *et al.*, 1991; Gutiérrez *et al.*, 1997; Nation *et al.*, 1999). In the USA the consumption of unpasteurized dairy products remains a concern (Center for Disease Control and Prevention, 2005; Rodwell *et al.*, 2008). As reported by the European Food Safety Authority (2011), the number of confirmed cases of human tuberculosis due to *M. bovis* in the EU increased slightly by 7.5 % (115 reported cases) in 2008 compared to 2007 (107 reported cases) (numbers for 2009 and 2010 not yet available). Five countries, Germany, Ireland, the Netherlands, Spain and the UK, accounted for 94.8 % of confirmed cases reported in 2008.

M. bovis and *M. caprae* cause a clinical syndrome in humans which is indistinguishable from that due to *M. tuberculosis*. Tuberculosis infections in humans are often reported without differentiation between infection by *M. tuberculosis*, *M. bovis* or *M. caprae*. For this reason the amount of cases due to *M. bovis* or *M. caprae* is probably underestimated; particularly in the developing world where sophisticated typing methods are deficient (Cosivi *et al.*, 1998; Ayele *et al.*, 2004). Moreover, the description of *M. caprae* as a separate species is relatively recent (Aranaz *et al.*, 2003), and before its elevation to species rank it was considered a subspecies either of *M. tuberculosis* or *M. bovis*, so that the estimate of infection due to *M. caprae* might be even more uncertain. Some authors speculated that *M. bovis* might cause up to 10-15% of the total of cases in these regions, while the overall percentage of tuberculosis due to *M. bovis* in industrialised countries ranged from 0.46 to 7.2% (Cosivi *et al.*, 1998; Ashford *et al.*, 2001).

In Great Britain it was estimated that 6% of the deaths in tuberculosis patients during the 1930's were caused by *M. bovis*, which was attributed to the high prevalence of tuberculosis in cattle (15-20%) (Hardie and Watson, 1992). Nowadays, in Great Britain no increase in human cases has been observed despite the resurgence of bovine tuberculosis; human infection is mostly due to contact to animals as observed by genotyping (Gibson *et al.*, 2004; Jalava *et al.*, 2007; Mandal *et al.*, 2011). Cotter and colleagues (1996) also suggest that transmission of *M. bovis* from animals to humans does not occur to a significant level in south-west Ireland. In Spain, a national survey observed that *M. bovis* and *M. caprae* cause 1.9% and 0.3% of the cases of human tuberculosis, respectively (Rodríguez *et al.*, 2009). Moreover, the genotypes isolated from the patients matched the most frequent genotypes found in animals in Spain, suggesting

an epidemiological link between infection in animals and humans. Contact with livestock was also believed the most likely source of infection in earlier studies (Gutiérrez *et al.*, 1997; Prodinger *et al.*, 2002). Reports from central-eastern Europe and from Italy confirm the presence of infection with *M. bovis*/*M. caprae* in humans (Erler *et al.*, 2004; Kubica *et al.*, 2003; Blaas *et al.*, 2003; Lari *et al.*, 2011). A recent study from Australia reports that *M. bovis* accounts for 0.2% of the cases in humans (Ingram *et al.*, 2010).

Surveys in the United States, Scandinavia and England report that reactivation of dormant lesions led to pulmonary disease in approximately 50% of the patients and also to infection involving the genitourinary tract (25%) and other non-pulmonary sites (25%) (Grange and Yates, 1994). Cosivi and colleagues (1998) observed that *M. bovis* mainly caused extra-pulmonary disease in humans in the developing countries and a later study from Tanzania reported that 10% of the extra-pulmonary cases were due to *M. bovis* (Zinsstag *et al.*, 2006). In a four-year survey from Tuscany (Italy) 3.7% of extra-pulmonary disease was caused by *M. bovis*, while this pathogen accounted for only 1.0% of the pulmonary cases (Lari *et al.*, 2009). In contrast, *M. caprae* is known to cause a variety of conditions in humans which include lung infection (Rodríguez *et al.*, 2009), lupus vulgaris and cutaneous tuberculosis (Meyer *et al.*, 2005; Fraile *et al.*, 2006; Tar *et al.*, 2009), urinary infection (Sintchenko *et al.*, 2006) and pericarditis (Blaas *et al.*, 2003).

It is widely believed that *M. bovis* and *M. caprae* are less virulent for human beings than *M. tuberculosis* and that human to human transmission is reduced (Kovalyov, 1989). This view remains controversial (Thoen and LoBue, 2007) since several cases of infection with person-to-person transmission were described in the past, mostly due to consumption of unpasteurized dairy products and close social contact (LoBue *et al.*, 2003; LoBue *et al.*, 2004a; LoBue *et al.*, 2004b; Thoen *et al.*, 2006; Evans *et al.*, 2007). Moreover, it is not concordant with the most important recent outbreak of *M. bovis* which occurred during the 1990ies in Spain (Rullán *et al.*, 1996; Guerrero *et al.*, 1997; Rivero *et al.* 2001). This outbreak was caused by an MDR *M. bovis* strain with a high mortality rate that affected over 100 patients in several hospitals and also patient contacts; all the patients were immunodeficient. Sporadic cases of infection with this strain are still observed (Robles *et al.*, 2002; Ramos *et al.*, 2004). Another case of human to human transmission of *M. bovis* has been reported from the UK within a cluster of six patients with common social links in an urban setting, where only one affected person had a history of zoonotic exposure (Evans *et al.*, 2007). Human to cattle transmission has been documented for *M. bovis* (Lesslie, 1962; Huitema, 1969; Lepper and Corner, 1983; Collins and Grange, 1987; Regassa *et al.*, 2008; Mandal *et al.*, 2011) and *M. tuberculosis* (Romero *et al.*, 2011).

Molecular characterisation has contributed substantially to the comparison

between human and animal isolates and therefore improved the knowledge of epidemiological relationships between infections. However, exchange of information has been hampered for a long time due to the lack of common strategies for public health and veterinary services. Fortunately the situation has improved over the last years, since it is now generally accepted that the control of zoonoses benefits both human and livestock (Zinsstag *et al.*, 2007).

5. Molecular typing methods

Molecular typing makes use of genetic markers in order to search for outbreak sources, to track epidemic or pandemic spread of particular strains or to reconstruct the evolution of a certain group of bacteria. The most currently utilised methods of strain identification have developed since 1990. Prior to this, phenotypic markers such as mycobacterial phage susceptibility and drug resistance patterns were used to distinguish between strains, but phagetyping (Richards, 1974) only provided limited strain differentiation and antibiograms are only useful for drug-resistant strains so that epidemiological investigations were limited. It was not until the 1990's that progress was made towards the standardisation of typing protocols in order to improve the possibilities of epidemiological investigations (van Embden *et al.*, 1993; Cousins *et al.*, 1998c). The implementation of modern genotyping methods contributed to the control and understanding of the pathogenesis of tuberculosis.

The major genotyping techniques used for strain differentiation of members of the MTBC can be classified in two categories (Figure 14): the whole genome techniques and the partial genome techniques (Durr *et al.*, 2000a). Whole genome techniques were the first to be described and have the advantage to use all the potential genetic information. However, these methods, including restriction endonuclease analysis (REA) (Collins and de Lisle, 1985) and pulsed field gel electrophoresis (PFGE) (Zhang *et al.*, 1992), are technically demanding, difficult to automate and therefore less popular than the techniques that only use part of the genome for typing. The partial genome techniques can be subdivided into (a) techniques targeting specific repeated sequences comprising insertion sequences (IS) (Collins and Stephens, 1991; Poulet and Cole, 1995), the direct repeat (DR) region (Kamerbeek *et al.*, 1997), polymorphic GC-rich sequences (Cousins *et al.*, 1998c) and tandem repeat loci (Frothingham, 1998), (b) a technique that uses random sequences, the random amplified polymorphic deoxyribonucleic acid (RAPD) analysis (Palittapongarnpim *et al.*, 1993), (c) typing aiming at house-keeping genes (MLST) (Maiden *et al.*, 1998), (d) deletion typing targeting RDs and (e) SNP typing (Figure 14).

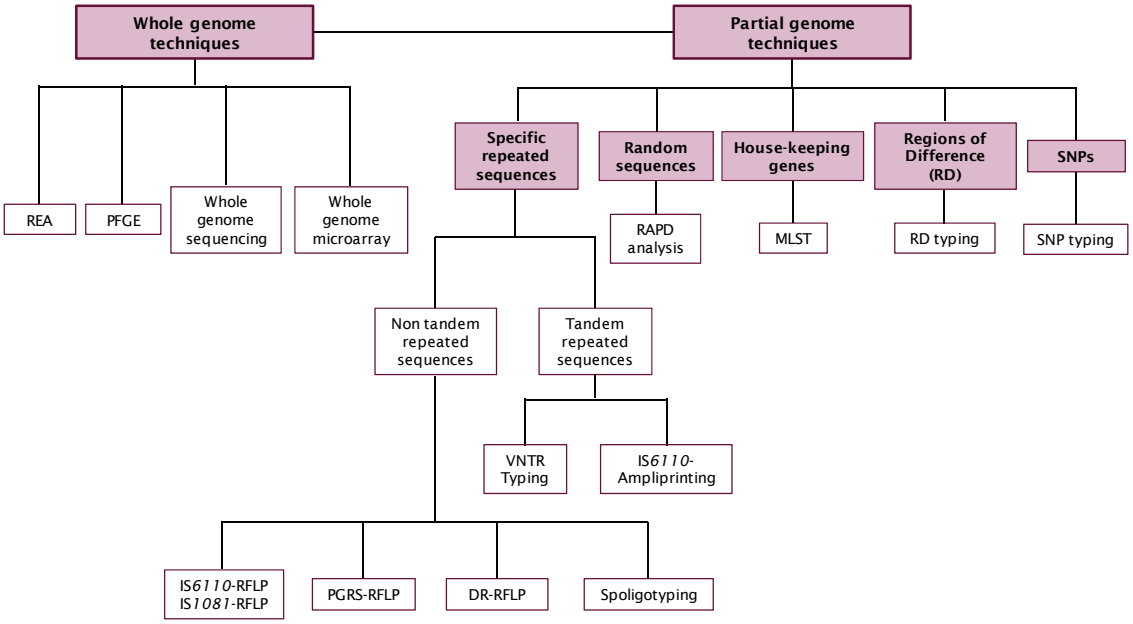


Figure 14. Overview over the most important techniques used for typing of the *Mycobacterium tuberculosis* complex. REA: restriction endonuclease analysis; PFGE: pulsed field gel electrophoresis; RAPD: Random amplified polymorphic deoxyribonucleic acid; MLST: multilocus sequence typing; VNTR: variable number tandem repeat; RFLP: restriction fragment length polymorphism; IS: insertion sequence; DR: direct repeat; PGRS: polymorphic GC-rich sequences; SNPs: single nucleotide polymorphims. Adapted from Durr *et al.* (2000a).

A critical point regarding the several typing techniques is the mutation rate. The mutation rate describes the pace at which molecular fingerprint patterns change and its knowledge is indispensable for the correct interpretation of molecular data for epidemiological or phylogenetic use (Reyes *et al.*, 2010; Grant *et al.*, 2008; Wirth *et al.*, 2008). If the mutation rate is high, an overestimation of epidemiologically unrelated tuberculosis cases is observed (Supply *et al.*, 2011). Furthermore, the analysis of the way variation occurs is essential to improve the efficacy of epidemiological purposes.

The most widely used techniques for molecular typing of members of the MTBC target the IS*6110*, the DR region and variable number tandem repeat (VNTR) loci (Mathema *et al.*, 2006; Collins *et al.*, 2011). While IS*6110*-RFLP is the gold standard for epidemiological studies in human tuberculosis, it never became established for molecular typing in animal tuberculosis due to the low copy number of IS*6110* elements in *M. bovis*.

5.1. Whole genome techniques

5.1.1. Restriction endonuclease analysis

Restriction endonuclease analysis (REA) was the first method developed for typing of *M. bovis* isolates (Collins and de Lisle, 1985) and uses three restriction

endonuclease enzymes, *Bst*EII, *Pvu*II and *Bcl*I, to cleave the DNA strands of whole genomic DNA at specific nucleotide sequences. This digest results in many small fragments which are separated by agarose gel electrophoresis. The fragment patterns allow for strain differentiation. REA allowed for the first time to determine whether livestock had become infected on the farm or infection had entered the farm through purchase of an already infected animal (Collins *et al.*, 1994a; Collins *et al.*, 1994b). This technique has been applied for epidemiological studies in New Zealand (Collins *et al.*, 1983) and Ireland (Collins *et al.*, 1994b), but it did not become generally accepted due to technical issues and difficulties with interpretation of the patterns (Collins *et al.*, 1993) and has only been used as part of the bovine tuberculosis control scheme in New Zealand.

5.1.2. Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) solved the problem of the excessive number of small DNA fragments encountered with REA using restriction enzymes which do not generate that many fragments. These fragments are too large to be separated by conventional agarose gel electrophoresis but can be detected when subjected to a pulsed electrical field. The technique was first implemented for *M. tuberculosis* isolates (Zhang *et al.*, 1992) and later set up for *M. bovis* BCG (Zhang *et al.*, 1995) and *M. bovis* (Feizabadi *et al.*, 1996). PFGE has not been established as a standard technique because it is difficult and labour-intensive.

5.1.3. Whole genome sequencing

The importance of the knowledge of gene and protein sequences for the scientific community is reflected by the exponential growth of data registered in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) over the past 15 years. Especially complete genomes (www.ncbi.nlm.nih.gov/Genomes/) continue to represent a rapidly growing segment of the database (Benson *et al.*, 2011). Since the first description of Sanger dideoxy terminator sequencing (Sanger *et al.*, 1977) the steady change of the sequencing technology entailed the need for infrastructure of robotics, bioinformatics, computer databases and instrumentation (Mardis, 2008a). Sanger's method has provided the backbone for DNA sequencing for the last 40 years and has allowed the development of high throughput genetic analysis by capillary-based sequencing, with the 96-capillary 3730xl DNA Analyzer (Applied Biosystems™) being the gold standard. High throughput next-generation sequencing (HT-NGS) was developed in order to substitute the initially time- and cost-intensive methods by analyzing millions of sequences in parallel rather than 96 at a time. At the moment there are three widely used platforms available for massively parallel DNA sequencing, that are also referred to as second-generation sequencing (Mardis, 2008a; Mardis, 2008b): the Roche 454 GS-FLX sequencer (Roche

Applied Science) based on the principle of pyrosequencing (Margulies *et al.*, 2005), the Illumina® genome analyzer (Illumina, Inc.) based on the concept of "sequencing by synthesis" (SBS) (Bentley, 2006) and the SOLiD™ (Sequencing by Oligo Ligation and Detection) sequencer (Applied Biosystems™) (Pandey *et al.*, 2008). An important advantage of HT-NGS is that relatively little DNA (5 µg) is needed. The sequence-ready libraries are obtained by ligating specific adapter oligos to both ends of DNA fragments generated by different front-processes. Before the sequencing of the fragments they undergo amplification by either emulsion PCR (Roche 454 GS-FLX and SOLiD) or bridge amplification (Illumina®). A recent development, the third generation sequencing, minimizes base sequence errors by skipping amplification and determining the sequence directly from a single DNA molecule (Schadt *et al.*, 2010). Platforms based on this method are the HeliScope™ sequencer (Helicos BioSciences Corporation) and the SMRT™ (single molecule real time) sequencer (Pacific Biosciences™) (Pareek *et al.*, 2011).

The availability of genome-wide information of single nucleotide to large sequence polymorphisms coupled with potent computational analysis tools is causing a revolution in genomics which will change the nature of current genetic experimentation (Mardis, 2008a). Of course, whole genome sequencing has also been applied to improve the studies of the mycobacteria and several genome sequences are available today (Table 4). Undoubtedly, the genome sequence of *M. tuberculosis* H37Rv (Cole *et al.*, 1998) was a breakthrough in molecular tuberculosis research, followed by the genome sequences of *M. bovis* AF2122/97 (Garnier *et al.*, 2003) and *M. bovis* BCG (Brosch *et al.*, 2007). Ongoing sequencing projects will deepen our insight not only into the evolution of the MTBC but also into the nature of tuberculosis infection.

Table 4. Published whole genome sequences of mycobacterial species sorted by species name and completion/creation date.

Species[a]	Strain	GenBank[b]	Completed[c]	Publication[d]
*M. africanum**	GM041182	FR878060	05/07/2011	-
M. avium subsp. *paratuberculosis*	K-10	AE016958	27/12/2001	Li *et al.*, 2005
M. avium	104	CP000479	29/11/2006	-
*M. bovis**	AF2122/97	BX248333	25/01/2001	Garnier *et al.*, 2003
M. bovis BCG*	Tokyo 172	AP010918	13/03/2009	Seki *et al.*, 2009
M. bovis BCG*	Pasteur 1173P2	AM408590	08/01/2007	Brosch *et al.*, 2007
M. canettii	CIPT 140010059	HE572590	14/07/2011	-
M. gilvum	PYR-GCK	CP000656	13/04/2007	-
M. leprae	TN	AL450380	02/10/2001	Cole *et al.*, 2001
M. leprae	Br4923	FM211192	10/01/2009	Monot *et al.*, 2009
M. marinum	M	CP000854	19/04/2008	Stinear *et al.*, 2008
M. smegmatis	MC2 155	CP000480	29/11/2006	-
*M. tuberculosis**	H37Rv	AL123456	07/09/2001	Cole *et al.*, 1998
*M. tuberculosis**	CDC1551	AE000516	02/10/2001	Fleischmann *et al.*, 2002
*M. tuberculosis**	H37Ra	CP000611	06/06/2007	Zheng *et al.*, 2008
*M. tuberculosis**	F11	CP000717	14/06/2007	-
*M. tuberculosis**	KZN 1435	CP001658	14/07/2009	-
*M. tuberculosis**	KZN 4207	CP001662.1	07/04/2011	-
*M. tuberculosis**	CCDC5079	CP001641	05/07/2011	-
*M. tuberculosis**	CCDC5180	CP001642	05/07/2011	-
*M. tuberculosis**	CTRI-2	CP002992	25/08/2011	-
M. ulcerans	Agy99	CP000325	04/12/2006	Stinear *et al.*, 2007
M. vanbaalenii	PYR-1	CP000511	28/12/2006	-

[a] Mycobacterial species (or subspecies). *, Member of the *Mycobacterium tuberculosis* complex.
[b] GenBank accession number.
[c] Date of creation/completion.
[d] First publication of the genome.

5.1.4. Whole genome microarray

The availability of whole genome sequence data for several strains of the MTBC (Table 4) enabled the use of the microarray technique for comparing a particular strain to sequenced reference strains (Butcher, 2004). Bacterial microarrays have been mainly used for comparative genomic hybridization (CGH), also referred to as comparative genomics (DNA microarray), and for comparison of gene expression, also named transcriptomics or expression profiling (RNA microarray). The basic principle is similar independent from the starting product (DNA or RNA). PCR products representing ORFs of the genes of the reference strain(s) are spotted onto glass slides, which remain the preferred solid support for spotted arrays; for this purpose the use of commercial microarray robots, that are able to place the spots precisely on the slides, has increased, e.g. RoboAmp 4200 robot (MWG Biotech) (Holloway *et al.*, 2002). The samples

(experimental and control sample) are amplified (PCR amplification for DNA and reverse transcription for RNA), labelled with fluorescent dyes (different dyes for experimental and control sample) and hybridised onto the microarray slides. For visualisation of the spots, the slides are introduced in a fluorescence reader. Subsequently, the spot intensities have to be quantified using adequate software, e.g. BlueFuse v. 3.5 (BlueGnome Ltd.). A microarray experiment generates thousands of data and constitutes a challenge for storing and processing these data; extensive equipment and software is therefore required (Bowtell, 1999; Holloway *et al.*, 2002). Widely used software for microarray data analysis is the GeneSpring™ package (Agilent Technologies) (Chu *et al.*, 2001; Garcia-Pelayo *et al.*, 2004).

Comparative genomics elucidate differences between members of the MTBC by surveying whole genomes and have improved our understanding of the pathogenesis and host-adaptation (Gordon *et al.*, 2009). A key advantage for comparative genomics of the MTBC is the 99.95% identity at nucleotide level (Cole *et al.*, 1998; Garnier *et al.*, 2003). DNA microarrays constitute a powerful CGH technique which allows the comparison of chromosomal differences regarding mycobacterial virulence and other host-determining factors (Behr *et al.*, 1999b; Inwald *et al.*, 2002). Chromosomal polymorphisms can also be suitable as markers for a distinct species or clonal complex (Bigi *et al.*, 2005; Garcia-Pelayo *et al.*, 2004; Müller *et al.*, 2009; Berg *et al.*, 2011; Smith *et al.*, 2011). Transcriptomics has been applied to study possible virulence factors by comparing expression profiles of virulent, avirulent and vaccine strains (Voskuil *et al.*, 2004; Blanco *et al.*, 2009; Aranday Cortes *et al.*, 2010). Although equipment for microarray analysis is not inexpensive, the analysis per se is reasonably cheap and only relatively small amounts of starting sample are required. In addition, the amount of providers of commercial microarray analysis is increasing (e.g. IMGM® Laboratories and SABiosciences™) so that it is no longer necessary to dispose of the equipment in order to obtain microarray data.

5.2. Partial genome techniques

5.2.1. Restriction fragment length polymorphism analysis

In order to improve the REA a new technique was developed which did not aim at the whole genome but at a specific region. Restriction fragment length polymorphism analysis (RFLP) makes use of the restriction enzyme *Pvu*II or *Alu*I followed by agarose gel electrophoresis and subsequent Southern blotting of the fragmented DNA onto nitro-cellulose or nylon filter. To visualise specific fragments of DNA, labelled markers are added which are complementary to only a fraction of the DNA of the isolate. Target regions that have been utilised for RFLP are ISs, GC-rich repeat sequences (PGRS) (Cousins *et al.*, 1998c) and the DR sequence (Cousins *et al.*,

1998a). The best differentiation of strains is obtained by labour-intensive combination of the different RFLP analyses (Skuce *et al.*, 1994; Gutiérrez *et al.*, 1995; Aranaz *et al.*, 1998).

5.2.1.1. Insertion sequences IS*6110* and IS*1081*

The first extensively used method for strain identification, RFLP (van Embden 1999), was based on the insertion element IS*6110*. Insertion sequences, as well as transposons, are mobile genetic elements which constitute a form of repetitive DNA in bacterial genomes. They were first described by Barabara McClintock as "controlling elements", chromosomal elements, able to move, that affect gene expression and provoke chromosomal rearrangement (McClintock, 1956). Twenty years later, when molecular genetics allowed the isolation of those mobile genetic elements, they were identified in *Escherichia coli* (Kleckner *et al.*, 1977) and led to the extensive use of transposons in molecular genetics.

All mobile elements share certain characteristics: they are discrete DNA fragments encoding their own transposition functions and are able to move from one site in the DNA strand to another independently of host recombination functions. Recombination by transposition can occur without homology of the transposon and the target sequence, and many mobile elements insert randomly, however, some prefer specific target sites, so-called hot spot regions (Hermans *et al.*, 1991). The potential mobility of IS elements constitutes a source of genome plasticity, as it is likely that they lead to chromosomal rearrangements as well as deletions (Mahillon and Chandler, 1998). There are two theories regarding IS elements from an evolutionary point of view. On the one hand, Charlesworth and colleagues (1994) hypothesised that the elements might be genomic parasites that cause mutations and subsequent loss of fitness to bacteria; on the other hand Blot (1994) postulates that IS elements are able to cause advantageous mutations and therefore contribute to the adaptive evolution of the bacteria.

IS*6110* is the best characterised IS element in the *M. tuberculosis* genome. This fragment of 1,361 bp is not present in mycobacteria other than members of the MTBC; it was also isolated and referred to as IS*986* and IS*987* before it was recognised as IS*6110* (Thierry *et al.*, 1990; Hermans *et al.*, 1991; Poulet and Cole, 1995). IS*6110*-RFLP uses the restriction enzyme *Pvu*II, a hybridisation probe specific to the right side of IS*6110* and standardised molecular weight markers (van Embden *et al.*, 1993). The development of computational analysis of the RFLP patterns facilitates intra- and interlaboratory comparison of the results (Heersma *et al.*, 1998; Kremer *et al.*, 1999).

Studies have also targeted IS*1081* for use in the MTBC (Collins and Stephens, 1991; van Soolingen *et al.*, 1991; Poulet and Cole, 1995). However, studies

applying IS*1081*-RFLP to *M. bovis* isolates resulted in limited or even no discrimination (Collins *et al.*, 1993; Skuce *et al.*, 1994; Aranaz *et al.*, 1998), except from *M. bovis* BCG which can be reliably recognised by IS*1081*-RFLP (van Soolingen *et al.*, 1992).

A weakness of these methods is the possible addition of insertion sites by replicative transposition of the IS. Nevertheless, RFLP based on the IS*6110* insertion sequence is the most widely used typing technique for *M. tuberculosis* strains, which harbour up to 20 copies of IS*6110* (McAdam *et al.*, 1990; Thierry *et al.*, 1990). When strains harbour less than six copies of IS*6110* as occurs in some low-copy-number *M. tuberculosis* strains and in most *M. bovis* strains, including *M. bovis* BCG, this technique lacks discrimination and is therefore hardly ever used for typing *M. bovis* strains (Collins *et al.*, 1993; Romano *et al.*, 1996; Aranaz *et al.*, 1998; Cousins *et al.*, 1998a; Costello *et al.*, 1999; Sun *et al.*, 2004; Michel *et al.*, 2008). Interestingly, some *M. bovis* isolates show higher copy numbers (Rigouts *et al.*, 1996; Liébana *et al.*, 1997; Aranaz *et al.*, 1998; Assiimwe *et al.*, 2009; Berg *et al.*, 2011). Berg and colleagues (2011) hypothesised that the general association of *M. bovis* with low copy numbers might have developed due to the global presence of a group of *M. bovis* strains that generally has only one copy of IS*6110* (Smith *et al.*, 2011).

5.2.1.2. Polymorphic GC-rich repeat sequences

With a GC content of 66% in their genome mycobacteria belong to the GC-rich bacteria (Cole *et al.*, 1998). Some regions, consisting of short, repetitive sequences, show a GC composition of up to 80% (Ross *et al.*, 1992; Poulet and Cole, 1995). These polymorphic GC-rich sequences (PGRS) can be exploited for molecular typing (Ross *et al.*, 1992; Cousins *et al.*, 1998c). Although this technique achieves high discriminatory levels especially when multiple copies of IS*6110* are absent (Cousins *et al.*, 1998a; 1998c), it has not become implemented because it is technically demanding.

5.2.1.3. Typing based on the direct repeat region

The direct repeat (DR) region (Groenen *et al.*, 1993; van Embden *et al.*, 2000) is one of the most important genomic targets used for molecular typing of MTBC isolates and spoligotyping is the most extensively applied technique for the exploitation of this locus (see section 5.2.2). However, it is also suitable for the use with RFLP (Cousins *et al.*, 1998a; Zumárraga *et al.*, 1999a). Although DR-RFLP achieves similar discrimination than spoligotyping, the latter remains the technique of choice because it is easier to perform and interpret.

5.2.2. Spoligotyping

Groenen and colleagues (1993) identified a clustered regularly interspaced palindromic repeat (CRISPR) region unique to the MTBC. They named it Direct Repeat (DR) region since it consists of multiple 36 bp DRs interspersed by unique sequences called spacers; the spacer length ranges from 25 to 41 bp (van Embden *et al.*, 2000). A DR plus its adjacent spacer is called a direct variant repeat (DVR). Since strains vary in the number of DVRs, the presence or absence of the spacers can be exploited for strain typing. Although 104 spacer sequences have been identified in the DR region, not all of them show reasonable polymorphism to be used for strain typing (van Embden *et al.*, 2000; Caimi *et al.*, 2001). Kamerbeek and colleagues (1997) developed today's standard protocol for spacer oligonucleotide typing (spoligotyping) based on 43 spacers. Spoligotyping is a simple, rapid and robust technique that permits high-throughput typing of MTBC isolates without the need to purify the DNA. Two primers are used to target the individual DRs and amplify the whole DR region followed by hybridisation of the fragments onto a blot membrane, which is covalently linked to oligonucleotides representing the spacer sequences, and subsequent visualization via chemiluminescence (Figure 11); the membranes can be either commercial (Ocimum Biosolutions) or self-made, with the latter option producing equivalent or better results. This reverse line blot hybridisation method produces data that are easy to store thanks to the translation of the patterns into a binary code (0, absence of spacer; 1, presence of spacer), and internationally used authoritative names (prefix SB followed by four digits or prefix SIT followed by one to four digits) for the spoligotype patterns can be obtained from the major websites (http://www.Mbovis.org; http://www.pasteur-guadeloupe.fr/tb/bd_myco.html).

Several spacers have been found to be potentially problematic, which was confirmed in a recent interlaboratory comparison where errors at spacers 14, 15, 18, 39 and 40 were most frequent (Abadia *et al.*, 2011). In the case of spacer 15 this might be due to a deletion of four nucleotides at the 5' end of the adjacent DVR (DVR26) which hinders the proper amplification of this spacer (van Embden *et al.*, 2000).

Recently, the membrane-based assay has been transferred to the Luminex® platform (Luminex Corp.), a microbead-based multianalyte profiling system (Cowan *et al.*, 2004). Spoligotyping on the Luminex platform offers many advantages over traditional spoligotyping. Firstly, turnaround time and labour involved are decreased and secondly, the reproducibility is increased. Although the spoligotyping assay is reproducible (>90%) (Kremer *et al.*, 1999), reproducibility can be influenced by the subjective interpretation of the hybridization signal, that changes with repeated use of the membrane, and by human error at the manual data entry (Cowan *et al.*, 2004). Both membrane- and human-related quality issues that can affect the spoligotyping results can be improved, or even avoided, with the microbead-based method (Zhang *et*

al., 2009; Abadia *et al.*, 2011). Unfortunately, a major disadvantage of Luminex® is the initial acquisition cost.

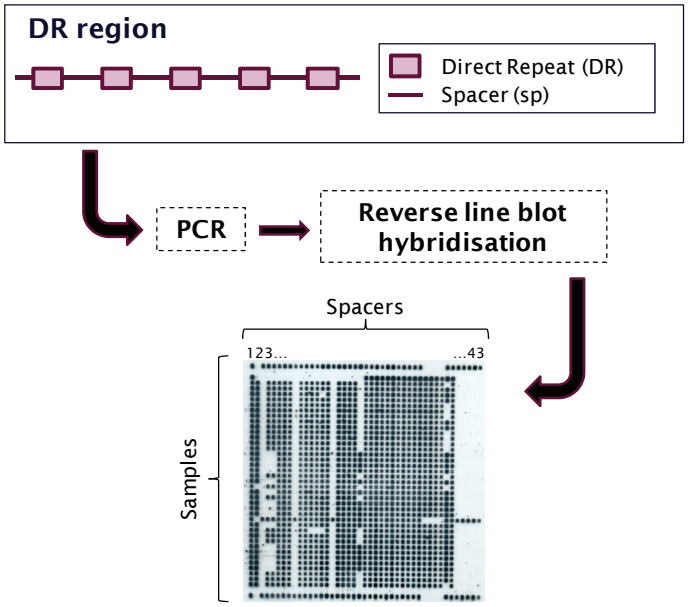


Figure 15. Scheme of the direct repeat (DR) region and the spoligotyping technique.

For strains with high IS*6110*-copy number (> 6) spoligotyping offers less discrimination than IS*6110*-RFLP, but spoligotyping is the undoubtedly the method of choice for IS*6110*-low-copy-number strains, such as *M. bovis* or some *M. tuberculosis* strains (Cousins *et al.*, 1998a; Thong-On et al, 2010). Several studies observed increased discrimination by combining spoligotyping and IS*6110*-RFLP (Cousins *et al.*, 1998a; Aranaz *et al.*, 1998; Roring *et al.*, 1998; Kremer *et al.*, 1999; Bauer *et al.*, 1999; Costello *et al.*, 1999; Soini *et al.*, 2001); however, RFLP is technically more demanding, time-intensive and high quantities of DNA are required so that spoligotyping is preferred. Since the implementation of spoligotyping for *M. bovis* (Aranaz *et al.*, 1996a; Roring *et al.*, 1998), the technique has been considered useful as a fast method for the characterisation of isolates and is applied at large scale for first-line typing (Smith *et al.*, 2003; Haddad *et al.*, 2004; Milian-Suazo *et al.*, 2008).

Moreover, spoligotyping allows for species differentiation based on the characteristic absence of certain spacers within the MTBC species (Aranaz *et al.*, 1996a; Kamerbeek *et al.*, 1997; Aranaz *et al.*, 1999; Niemann *et al.*, 2000c; Viana-Niero *et al.*, 2001; Smith *et al.*, 2009b). For example, spacer 3, 9, 16 and 39-43 are always missing in *M. bovis* strains, while *M. caprae* is characterised by the loss of spacers 1, 3-16, 28 and 39-43 (Table 5). Characteristic spacer deletions are often referred to as spoligotype signatures (Streicher *et al.*, 2007) and are able to provide additional information about lineage or clonal complex membership within the different species (Niemann *et al.*,

2000c; Kremer *et al.*, 2004; Müller *et al.*, 2009; Berg *et al.*, 2011; Kato-Maeda *et al.*, 2011; Smith *et al.*, 2011). Yet, it has to be considered that spacer deletions might appear in phylogenetically unrelated strains due to convergent evolution, also known as homoplasy (Warren *et al.*, 2002).

Table 5. Spoligotyping patterns for selected members of the *Mycobacterium tuberculosis* complex.

Species	Spoligotype pattern (spacers 1-43)	Absence of spacers
M. tuberculosis	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■	34
M. africanum	■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	8, 9, 39
M. pinnipedii	□□□■■■■□□□□□□□□□□□□□□□□■■■■□□■■■■■■■■■■□□□□□	1-3, 8-22, 27, 28, 39-43
M. microti	□□□■■■■□□□□□□□□□□□□□□□□■■■■■■■■■■■■■■■□□□□□	1-3, 8-22, 39-43
M. caprae	□■□□□□□□□□□□□□□□□■■■■■■■■■■□■■■■■■■■■■□□□□□	1, 3-16, 28, 39-43
M. bovis	■■□■■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	3, 9, 16, 39-43

■ Presence of spacer.
□ Absence of spacer.

Variation in the DR region is attributable to either homologous recombination between adjacent DRs or transposition of the insertion sequence IS*6110*, which is almost invariably present in this region (Hermans *et al.*, 1991; van Embden *et al.*, 2000). Although the order of DVRs was found to be strongly conserved, duplications of DVRs have been observed which are probably due to homologous recombination and/or slippage during DNA replication (van Embden *et al.*, 2000). The evolution of the DR region is unidirectional, occurring by single spacer deletions or loss of contiguous spacer sequences (Fang *et al.*, 1998; van Embden *et al.*, 2000) and the different DVRs can apparently be deleted randomly. Nevertheless, the region between DVR 1 and DVR 24 (5' of the IS*6110* element) has been identified as a hot spot for deletions (Warren *et al.*, 2002). The unidirectional evolution of the DR region has facilitated studies on the variation of isolates of a single outbreak, of subsequent isolations from the same patient, of variation due to subculturing of strains, as well as phylogenetical studies (Zhang *et al.*, 1995; van Embden *et al.*, 2000; Aranaz *et al.*, 2004b; Aga *et al.*, 2006; Smith *et al.*, 2009a).

Van der Zanden (2002) postulated that the use of the 43 selected spacers might not be appropriate for *M. bovis* isolates since these spacers were chosen paying particular attention to discrimination in *M. tuberculosis* isolates. A possible solution to this problem was offered by the second-generation spoligotyping membrane that complimented the standard membrane in order to assess the whole 104 spacers and

achieved considerably improved discrimination (van der Zanden *et al.*, 2002). A different second-generation membrane with 25 additional spacers was developed to specifically assess its usefulness for typing Spanish *M. bovis* and *M. caprae* isolates; the additional spacers improved the strain differentiation of the *M. bovis* but not of *M. caprae* isolates (Javed *et al.*, 2007), which commonly show less variation in the DR locus than *M. bovis* isolates. A further limiting factor of spoligotyping is its inability to detect mixed infections, because the pattern obtained in this case corresponds to the cumulative presence of spacers (Romero *et al.*, 2008; Cohen *et al.*, 2011). This is of importance when samples are pooled before culturing, especially when different strains are present in different organs which have so far only been described in human tuberculosis (García de Viedma *et al.*, 2003).

The discrimination of *M. bovis* isolates achieved with spoligotyping varies strongly between different countries and even geographical regions. While large population surveys using spoligotyping in France (Haddad *et al.*, 2001), Portugal (Duarte et la., 2008), Italy (Boniotti *et al.*, 2009) and South Africa (Michel *et al.*, 2008) revealed satisfactory discrimination of *M. bovis/M. caprae* isolates, large scale spoligotyping in Australia (Cousins *et al.*, 1998a), the RoI (Costello *et al.*, 1999), Northern Ireland (Skuce *et al.*, 2005) and Great Britain (Hewinson *et al.*, 2006) was hampered because of low discriminatory indices (Table 6).

Table 6. Spoligotyping data from population surveys conducted in Europe and overseas. - , no data available.

Country	No. of isolates	*M. bovis* profiles[a]	*M. caprae* profiles[b]	Prevalence of frequent *M. bovis* spoligotypes (%)	Prevalence of *M. caprae* spoligotypes (%)	Animal species[c]	h[d]
Great Britain[1]	9,839	34	-	SB0140 (35.9) SB0263 (23.6) SB0274 (13.0)	-	C	0.79
Northern Ireland[2]	461	14	-	SB0140 (> 60) SB0142 (20) SB0263 (8)	-	C, B, O	0.53
Republic of Ireland[6]	452	20	-	SB0140 (51.8) SB0130 (15.3)	-	C, B, Wb, D, S, Sw, G	0.61[e]
France[3]	1,349	156	5	SB0120 (26.0) SB0121 (12.0) SB0134 (-)	SB0415 (-) SB0416 (-) SB0418 (-) SB0835 (-) SB0866 (-)	C, G, D, S, Sw, O	-
Portugal[4]	293	28	1	SB0121 (26.3) SB0119 (9.4) SB0886 (7.5)	SB0157 (2.4)	C, G, D, Wb	0.90
Italy[5]	1,560	81	7	SB0120 (54.6) SB0134 (5.7) SB0841 (4.8)	SB0418 (3.5) SB0416 (1.5) SB0866 (0.7) SB1885 (0.1)	C	0.70
Czech Republic and Slovakia[7]	21	6	1	SB0120 (33.3) SB0961 (9.5)	SB0418 (42.9)	C, D, Cb	0.79[e]

[1] Hewinson et al., 2006; [2] Skuce et al., 2005; [3] Haddad et al., 2001; [4] Duarte et al., 2008; [5] Boniotti et al., 2009; [6] Costello et al., 1999; [7] Pavlik et al., 2002a.
[a] Number of spoligotype patterns corresponding to *M. bovis*.
[b] Number of spoligotype patterns corresponding to *M. caprae*.
[c] Animal origin of the isolates : C = cattle, B = badger, Bu = buffalo, Cb = capybara, D = deer, G = goat, S = Sheep, Sw = swine, Wb = wild boar, H = human, O = other (not described).
[d] Diversity index (h) of Hunter and Gaston (1988).
[e] Estimation based on the published data.

Country	No. of isolates	*M. bovis* profiles[a]	*M. caprae* profiles[b]	Prevalence of frequent *M. bovis* spoligotypes (%)	Prevalence of *M. caprae* spoligotypes (%)	Animal species[c]	h[d]
Australia[8]	211	20	-	SB0140 (72.5) SB1030 (12.8) SB0487 (3.3)	-	C, O	-
South America[9]	224	41	-	SB0140 (42.8) SB0130 (5.3) SB0131 (4.4)	-	C, D, Sw, Bu, O	0.79
South Africa[10]	90	12	-	SB0131 (7.0) SB0130 (5.6) SB0140 (5.6)	-	C	0.91
Cameroon[11]	75	10	-	SB0944 (62.7) SB1461 (10.7) SB1460 (8.0)	-	C	0.59 [e]
Chad[11]	65	13	-	SB0944 (40.0) SB1025 (20.0) SB0951 (10.8)	-	C	0.79 [e]
Mali[11]	20	7	-	SB0300 (40.0) SB0134 (30.0) SB1410 (10.0)	-	C	0.77[e]
Ethiopia[12]	48	12	-	SB1176 (41.2) SB0133 (14.1) SB0912 (11.8)	-	C	0.81
Nigeria[13]	180	34	-	SB0944 (45.6) SB1027 (13.9) SB1025 (4.4)	-	C	0.76[e]

[8] Cousins et al., 1998a; [9] Zumárraga et al., 1999 (includes strains from Argentina, Costa Rica, Mexico, Uruguay, Paraguay and Brasil); [10] Michel et al., 2008;[11] Müller et al., 2009; [12] Biffa et al., 2011; [13] Cadmus et al., 2011.

[a] Number of spoligotype patterns corresponding to *M. bovis*.

[b] Number of spoligotype patterns corresponding to *M. caprae*.

[c] Animal origin of the isolates : C = cattle, B = badger, Bu = buffalo, Cb = capybara, D = deer, G = goat, S = Sheep, Sw = swine, Wb = wild boar, H = human, O = other (not described).

[d] Diversity index (h) of Hunter and Gaston (1988).

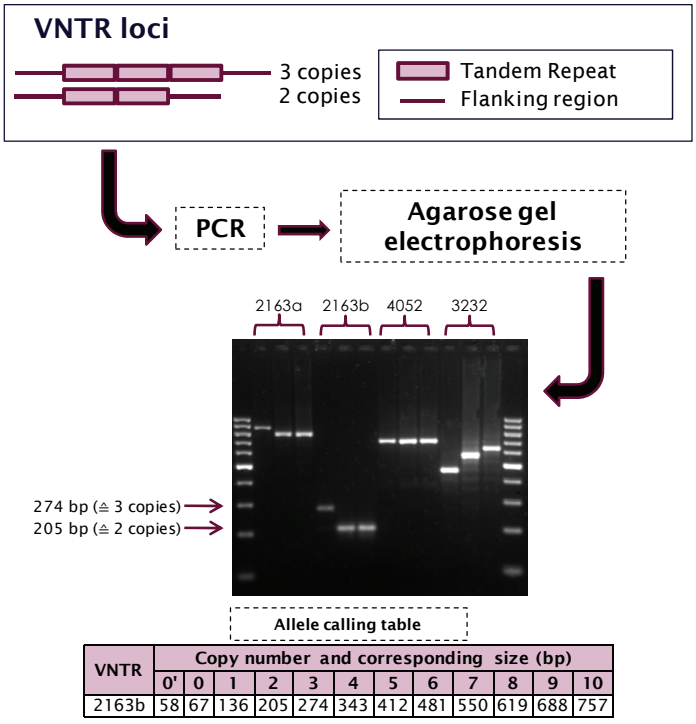
[e] Estimation based on the published data.

5.2.3. Variable number tandem repeat typing

Variable number tandem repeat (VNTR) typing aims at genetic loci that are distributed all over the genome; these loci contain variable numbers of repeated sequences (tandem repeats). Tandem repeats, also called microsatellites, are the basis for forensic analysis and paternity testing. In fact, the best characterised repetitive sequences are the eukaryotic *Alu* sequences which represent 3 to 6% of the human DNA interspersed throughout the genome (Jelinek *et al.*, 1980; Schmid and Jelinek, 1982). Tandem repeats have also been successfully used for fingerprinting of bacterial genomes thanks to extensive polymorphism in the copy number of the repeats (Versalovic *et al.*, 1991). VNTR typing is also known as multilocus variable-number tandem repeat analysis (MLVA) of other pathogenic bacteria and combines many advantages being cheap, quick and easy to perform and yielding unambiguous results (Lindstedt, 2005; van Belkum, 2007). VNTR typing is based on PCR amplification of the targeted loci with specific primer pairs followed by gel electrophoresis (Figure 11). The use of automatic sequencers to assess the exact size of the amplified fragments has also become established in many research and diagnostic laboratories and enables automated high-throughput genotyping (Supply *et al.*, 2001). For interpretation of the results, the use of an allele calling table that relates the different band sizes to the corresponding number of repeats for each locus is indispensable (Figure 16).

The first VNTR locus identified in the *M. tuberculosis* genome consisted of a 75 bp tandem repeat located within a large open reading frame (ORF) (Goyal *et al.*, 1994). The implications of VNTR loci located within ORFs are not yet understood, but the fact that the DNA of the repeat is translated during expression of the ORF, leading to size variation in the protein, hints at a possible functional role. A subsequent study using VNTR typing of MTBC isolates including the previously identified locus plus five novel tandem repeats named the loci exact tandem repeats (ETR) A to F; these ETRs range in size from 53 to 79 bp (Frothingham and Meeker-O'-Connell, 1998). In recent years several new loci have been described and some of them are also located within ORFs, for example QUB11a, QUB11b, QUB18, QUB23, QUB26 (Skuce *et al.*, 2002). All the loci that are located within coding regions have repeat sizes that are multiples of three. Most dispersed repeats are found in intergenic regions, so that they are thought not to have any functional implication in the bacterial genome. Supply and colleagues (1997) designated these loci mycobacterial interspersed repetitive units (MIRUs) which range in size from 46 to 101 bp. Since the use of the MIRUs is widespread, VNTR typing of mycobacteria is often referred to as MIRU-VNTR typing. The total number of MIRUs per genome is estimated to be about 40 to 50. It is noteworthy that some of the intergenic loci are located upstream of ORFs in the same orientation, for example ETR-D (MIRU 4) which was found within the intergenic region of an operon encoding a mycobacterial

two-component system (Supply *et al.*, 1997). It has been postulated that variations in the number of tandem repeats might affect the expression of downstream genes (Magdalena *et al.*, 1998). Generally, since the position of the loci is not always conserved among different bacterial species, they probably do not influence the function of a given gene, but rather are important for evolution of chromosome structure and DNA rearrangement, such as tandem duplications, chromosomal deletions and inversions (Petes and Hill, 1988).



VNTR	Copy number and corresponding size (bp)											
	0'	0	1	2	3	4	5	6	7	8	9	10
2163b	58	67	136	205	274	343	412	481	550	619	688	757

Figure 16. Scheme of variable number tandem repeat (VNTR) loci and VNTR typing.

Many additional VNTR loci have been reported since the first description of tandem repeats (Smittipat *et al.*, 2000; Smittipat *et al.*, 2005) and it soon became clear that standardisation of the nomenclature was necessary. Smittipat and colleagues (2005) suggested the use of "VNTR" followed by four digits corresponding to the locus position on *M. tuberculosis* H37Rv. Nonetheless, the different loci aliases are still widely used (ETR = exact tandem repeat; MIRU = mycobacterial interspersed repetitive unit; QUB = Queen's University Belfast). Many efforts have been made to standardise the typing protocol and especially of the use of certain VNTR loci in order to enable interlaboratory comparison of the results (Allix *et al.*, 2006; Supply *et al.*, 2006; Supply, 2006). The selection of certain sets of loci for VNTR typing is based on studies on their specificity, sensitivity and reproducibility (Kremer *et al.*, 1999; Supply *et al.*, 2001; Kremer *et al.*, 2005). Several loci, for example VNTR3232 and VNTR2163a, have been described as hypervariable due to a higher mutation rate regarding allelic diversity in *M. tuberculosis*

(Supply *et al.*, 2006). The mutation rate determines the discriminating capacity of a locus and there are considerable variations between the different loci. However, this issue remains controversial (Reyes and Tanaka, 2010; Supply *et al.*, 2011) since the influence of the particular setting (Hilty *et al.*, 2005), the mycobacterial species, for example *M. bovis* (McLernon *et al.*, 2010; Lari *et al.*, 2011), or even the lineage (Velji *et al.*, 2009) might generate conflicting results for the best choice of VNTR loci. An overview over the different suggestions for the use of VNTR loci is represented in Table 7.

Studies in order to determine an ideal set of VNTR markers for *M. bovis* are less abundant than studies on *M. tuberculosis*, nevertheless VNTR typing has been used in many countries for epidemiological studies (Roring *et al.*, 2002; Le Flèche *et al.*, 2002; Skuce *et al.*, 2002; Aranaz *et al.*, 2004a; Roring *et al.*, 2004; Skuce *et al.*, 2005; Allix *et al.*, 2006; Smith *et al.*, 2006b; Romero *et al.*, 2008; Boniotti *et al.*, 2009; Duarte *et al.*, 2010; Richomme *et al.*, 2010). In Europe, a group of scientists from laboratories investigating bovine tuberculosis have agreed on a consensus of six VNTR loci for the use with *M. bovis* (Supply, 2006); however, these loci are still not generally used which often hampers interlaboratory exchange. According to several reports, among the most discriminatory markers for *M. bovis* are QUB3232, ETR-A, ETR-B, QUB11a, QUB11b and QUB26 (Roring *et al.*, 2002; Hilty *et al.*, 2005; Romero *et al.*, 2008; Boniotti *et al.*, 2009; Lari *et al.*, 2011).

The use of VNTR typing for *M. caprae* isolates is even less frequent. Prodinger and colleagues (2005) analysed a large panel of *M. caprae* isolates by spoligotyping and VNTR typing with 12 MIRU markers (MIRU2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40). The best discrimination was found with MIRU 4, 10, 16, 26 and 31 which is not equivalent to the findings in *M. bovis* (Prodinger *et al.*, 2005). Generally, the same VNTR loci are assessed in both *M. caprae* and *M. bovis* isolates which might not offer optimised discrimination.

Table 7. Discriminatory variable number tandem repeat (VNTR) markers for typing of members of the *Mycobacterium tuberculosis* complex.

Locus VNTR	VNTR Alias	Length (bp)	(Frothingham y Meeker-O'Connell, 1998)[1] 6 loci	(Roring *et al.*, 2004)[2] 10 loci	(Prodinger *et al.*, 2005)[3] 5 loci	(Hilty *et al.*, 2005)[2] 10 loci	(Supply *et al.*, 2006)[4] 24 loci	(Supply *et al.*, 2006)[4] 15 loci	(Supply *et al.*, 2006)[4] 12 loci	(Allix *et al.*, 2006)[2] 9 loci	(Allix *et al.*, 2006)[2] 6 loci	(Supply, 2006)[2] 6 *loci*	(Boniotti *et al.*, 2009)[2] 13 loci	(Lari *et al.*, 2011)[2] 8 loci	(Cunha *et al.*, 2011a)[2,3] 8 loci
154	MIRU 2	53					X								
424	Mtub04	51					X	X	X						
577	ETR-C	58	X			X	X	X	X	X				X	X
580	MIRU 4 ETR-D	77	X		X	X	X	X		X		X		X	X
802	MIRU 40	54					X	X	X				X		
960	MIRU10	53			X		X	X	X						
1612	QUB23	21											X		
1644	MIRU 16	53			X	X	X	X	X						
1895	QUB1895	57		X									X		
1955	Mtub21	57					X	X	X						
2059	MIRU 20	77				X	X								
2163a	QUB11a	69		X						X	X	X	X		X
2163b	QUB11b	69		X			X	X	X	X	X		X	X	X
2165	ETR-A	75	X	X		X	X	X	X	X	X	X	X	X	X
2347	Mtub29	57					X								
2401	Mtub30	58					X	X						X	
2461	ETR-B	57	X	X		X	X			X	X	X	X		X
2531	MIRU 23	53					X								
2687	MIRU 24	54		X			X								
2996	MIRU 26	51		X	X	X	X	X	X	X	X		X		X
3007	MIRU 27 QUB5	53				X	X								
3155	QUB15	54											X		
3171	Mtub34	54					X								
3192	MIRU 31 ETR-E	53	X		X	X	X	X	X				X	X	
3232	QUB3232	56/57		X		X				X	X	X	X		X
3239	ETR-F	79	X												
3336	QUB3336	59		X									X		
3690	Mtub39	58					X	X	X						
4052	QUB 26	111		X			X	X	X			X	X	X	
4156	QUB4156	59					X	X		X				X	
4348	MIRU 39	53					X								

[1] *M. tuberculosis* complex; [2] *M. bovis*; [3] *M. caprae*; [4] *M. tuberculosis* complex but focused on *M. tuberculosis*.

VNTR typing has been useful for epidemiological studies and, in contrast to spoligotyping, it is able to identify mixed infection (Romero *et al.*, 2008). Double bands at a given locus hint at mixed infection and a possible case of microevolution, but it should also be taken into account that cross-contamination at sample-taking or in the

laboratory is a possible explanation for this finding. The use of VNTRs in phylogenetic studies is not as straightforward as the use of spoligotyping, because the evolution of VNTR loci is not unidirectional; repeats can be lost but also acquired so that the direction of transmission and evolution is more difficult to estimate (Arnold *et al.*, 2006). Yet, the use of VNTR typing as integral part of the bovine tuberculosis control programmes (Smith *et al.*, 2003; Boniotti *et al.*, 2009, Duarte *et al.*, 2010), generates a high amount of data which may be exploited to disclose the evolution and predominance of strains at global level.

5.2.4. IS*6110*-Ampliprinting

Ampliprinting targets the major polymorphic tandem repeat (MPTR) sequence (Hermans *et al.*, 1992) which is similar to the DR region, consisting of 10 bp direct repeats separated by 5 bp unique spacers. The MPTR has been identified in atypical mycobacteria and possesses limited polymorphism regarding the MTBC. Nevertheless, satisfactory results can be achieved in combination with the IS*6110*, named IS*6110*-ampliprinting, which uses the variable distance between IS*6110* and the copies of MPTR sequences (Plikaytis *et al.*, 1993). Since the results obtained with ampliprinting vary considerably in discrimination and reproducibility (Gutiérrez *et al.*, 1995; Glennon *et al.*, 1997; Kremer *et al.*, 1999), it is not applied as a routine method.

5.2.5. Random amplified polymorphic deoxyribonucleic acid analysis

Random amplified polymorphic deoxyribonucleic acid (RAPD) analysis is a simple PCR-based fingerprinting technique and has been widely used for typing bacteria (van Belkum, 1994). It was first described for typing of *M. tuberculosis* isolates by Palittapongarnpim and colleagues (1993) and has rarely been applied to *M. bovis*, apart from a study from Ireland in which only poor discrimination was obtained (Glennon *et al.*, 1997) and a study from Mexico (Milian-Suazo *et al.*, 2000). Due to problems with the reproducibility of the technique it could not establish itself for typing MTBC members.

5.2.6. Multilocus sequence typing

Multilocus sequence typing (MLST) aims at the subdivision of microbes on the basis of neutral sequence diversity (Maiden *et al.*, 1998). MLST is used for characterising isolates of bacterial species by sequence analysis of usually seven house-keeping genes. For this purpose, approximately 450-500 bp of internal fragments of each gene are amplified and sequenced. For each gene the different sequences are assigned as distinct alleles so that for every isolate an allelic profile or sequence type (ST) can be obtained by combining the alleles at each locus. House-keeping genes accumulate variation very slowly what makes them likely to be selectively neutral.

Although only a small number of alleles can be identified within the population by using this type of variation, high levels of discrimination are achieved by analyzing many loci. An advantage of this technique is that the data can be easily stored in large databases, presenting a powerful resource for global epidemiology. Most bacterial species have sufficient variation within house-keeping genes to provide many alleles per locus (Ibarz Pavón and Maiden, 2009). However, in the case of genetically monomorphic bacteria, as are the MTBC members (Sreevatsan *et al.*, 1997), the achieved levels of discrimination are insufficient (Achtman, 2008). To increase discrimination it might be necessary to characterize other than housekeeping genes such as genes encoding antigens and antibiotic-resistance determinants or the location of insertion sequences (Urwin and Maiden, 2003).

5.2.7. RD typing

RDs (see sections 2) can be exploited for species differentiation, e. g. RD9 which differentiates all other MTBC members from *M. tuberculosis* and *M. canettii* because it is only present in the latter species and RD4 which is absent from all isolates of *M. bovis*. As a result RDs have been used as polymerase chain reaction (PCR) targets for a quick species identification and further RDs have been reported more recently, such as RD2^{seal} (Bigi *et al.*, 2005), RD1^{mic} (Brodin *et al.*, 2002) and RD1^{das} (Mostowy *et al.*, 2004a). Different approaches have been described proposing the use of three primers, two flanking and one internal (Talbot *et al.*, 1997; Mostowy *et al.*, 2002), or four primers, two flanking and two internal primer pairs (Brosch *et al.*, 2002; Smith *et al.*, 2009b), to assess the presence or absence of the RDs. Several combinations of the different PCRs have been recommended for species identification (Parsons *et al.*, 2002; Huard *et al.* 2003; Huard *et al.*, 2006). Moreover, due to the unidirectional evolution of the RDs (Behr *et al.*, 1999; Gordon *et al.*, 1999) these markers are useful for the reconstruction of the evolution of the MTBC and to define clonal groups within the MTBC host-adapted members (Gagneux and Small, 2007; Reed *et al.*, 2009; Berg *et al.*, 2011; Smith *et al.*, 2011) (section 3.3).

5.2.8. Single nucleotide polymorphism typing

SNP typing is useful for species differentiation as described above (section 2, Table 2), but it has also become a powerful tool for the identification of distinct lineages in *M. tuberculosis* (Gutierrez *et al.*, 2005; Filliol *et al.*, 2006; Gagneux and Small, 2007; Hershberg *et al.*, 2008; Abadia *et al.*, 2010; Schürch *et al.*, 2011) and *M. bovis* (Smith *et al.*, 2006b; Garcia-Pelayo *et al.*, 2009) (section 3.2). The major disadvantage of SNP typing is that it relies on a two-step process, either PCR and sequencing or PCR and restriction endonuclease analysis, which is cost- and time-intensive and therefore its use

as a routine typing method is limited. However, SNP typing is needed for in-depth phylogenetic analyses.

5.3. Molecular epidemiology and databases

From the early stages of the implementation of molecular fingerprinting, it has been recognized as an important contribution to a more refined understanding of infectious disease epidemiology (Tompkins, 1992; van Embden *et al.*, 1993). Since then, molecular fingerprinting of MTBC isolates has been used as a powerful tool for the monitoring of disease transmission, the detection of spread of tuberculosis [even transcontinental (Long *et al.*, 1999)] and tuberculosis outbreaks (Bifani *et al.*, 1999). Especially against the background of the emergence of multidrug-resistant (MDR) tuberculosis molecular tracing is of greatest relevance (Mathema *et al.*, 2006). Notwithstanding, molecular typing of tuberculosis in livestock is also of outstanding importance in the light of its economic burden and public health polemic (Durr et al, 2000b). In developing countries where public resources for molecular typing are scarce, control strategies are seriously hampered, often leading to uncoordinated efforts and subsequent failure (Marcotty *et al.*, 2009). The precise knowledge of the epidemiology allows analysis of population dynamics and genetics, and improvement of the monitoring of tuberculosis control programmes (Neill *et al.*, 2005).

The first databases, created by the Institut Pasteur Guadeloupe, were focused on human tuberculosis and included information on spoligotyping results of a limited number of *M. tuberculosis* isolates from the Caribbean, Europe and the USA (Sola *et al.*, 1999). By systematically collecting published spoligotypes the initial 610 spoligotypes were increased to a total of 3,319 strains with 799 distinct spoligotype (Sola *et al.*, 2001). The third update, the so-called SpolDB3, achieved a better representation of the worldwide *M. tuberculosis* diversity (Filliol *et al.*, 2001; Filliol *et al.*, 2003). The fourth and latest update, SpolDB4, provides a higher resolution image of the worldwide *M. tuberculosis* genome diversity and thus, enables studies of epidemiology and population genetics (Brudey *et al.*, 2006). This database currently contains 39609 entries from 121 countries which cluster in 1939 different spoligotype patterns (Spoligotype International Type, SIT) (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html, consulted on 19th August 2011). The database also includes MIRU-VNTR typing results for 12 different MIRU loci [(MIRU 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40)] (Supply *et al.*, 2001) where the MIRU-VNTR type is represented by 12 digits (VNTR International Type, VIT).

A new, freely accessible database, MIRU-VNTRplus (http://www.miru-vntrplus.org/MIRU/index.faces) was created with a similar purpose (Allix-Béguec *et al.*, 2008; Weniger *et al.*, 2010). Apart from the spoligotyping pattern and a 24-locus, 15-locus or 12-locus MIRU-VNTR profile (Table 7) it additionally includes information on

single-nucleotide- and large-sequence-polymorphisms leading to optimal phylogenetic identification. Users of MIRU-VNTRplus can freely submit strains for analysis without adding the analysed data to the reference database. The analysis includes the comparison of the data with reference strains and calculation of distance coefficients and the creation of dendrograms using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) or Neighbour-Joining (NJ) clustering algorithms. Unfortunately, the amount of *M. bovis* isolates and in general isolates of animal origin in this database is very low compared to the number of human data, a fact that complicates data exchange between the public health and the veterinary sector.

In response to the need of an international nomenclature for spoligotypes of animal origin, in 2003 the *M. bovis* Spoligotype Database (http://www.Mbovis.org/) was created which is hosted on Animal Health Veterinary Laboratories Agency [(AHVLA) Weybridge, UK] servers and supported by the Department for Environment, Food and Rural Affairs (DEFRA) (Smith and Upton, 2011). This database assigns authoritative names to the binary spoligotyping pattern of MTBC members that lack the RD9 (*M. africanum*, oryx bacillus, dassie bacillus, *M. microti*, *M. pinnipedii*, *M. caprae*, *M. bovis* and *M .bovis* BCG). The authoritative name consists of a prefix followed by four digits (SBxxxx), the SB number. The nomenclature consisting of six blocks of two digit hexadecimal numbers (HEX code), e.g. 1111111 1101110 1111110 1111111 11111000 1111101 is called 7F-6E-7E-7F-F8-7D (Dale *et al.*, 2001), is not generally used and has been removed from the website in 2011. To date, the website contains 2025 different spoligotypes along with the corresponding information about the country where it was first isolated, the date of isolation and the person who submitted the pattern (consulted on 19th August 2011). Additionally, the animal origin, geographical region, corresponding publications or any other information related to the pattern can be included. The implementation of a common nomenclature has provided an easy way to compare spoligotyping results between laboratories and hence is of great value for the scientific community.

A manuscript that reviews the molecular typing methods for *M. bovis* and *M. caprae* isolates is in preparation and will be submitted with the title “An update of molecular typing of *Mycobacterium bovis* and *Mycobacterium caprae* and its relevance for epidemiological studies”.

Objectives and organisation of the thesis

Objectives and organisation of the thesis

The eradication of bovine tuberculosis is an important aim at European level and the EU member states are responsible for the adequate design and implementation of National Eradication Programmes. *M. bovis* and *M. caprae* are the main causative agents of animal tuberculosis in Spain and not only affect cattle and goats but a wide range of animal species and humans. In order to improve the national eradication campaign it is necessary to gain better insight into the epidemiology of these pathogens. In Spain the importance of both bovine and caprine tuberculosis is recognised and the role of wildlife as a reservoir of the disease is also considered crucial for the success of the eradication programme. Since the establishment of standardised molecular typing techniques, molecular epidemiology has become a pillar in eradication programmes worldwide as it contributes to a better understanding and monitoring of the disease.

The aim of this PhD thesis is the application of molecular typing techniques at national level in order to evaluate the epidemiological situation in Spain within the European context. Therefore several objectives have been addressed which will be exposed independently in the following chapters:

Chapter I:	Molecular demography of *Mycobacterium bovis* and *Mycobacterium caprae* in Spain
Chapter II:	Molecular typing as a tool in tracking outbreaks caused by *Mycobacterium bovis*
Chapter III:	The Spanish national database of animal tuberculosis - mycoDB.es
Chapter IV:	Phylogeny of *Mycobacterium bovis* in the Iberian Peninsula

Chapter I: Molecular demography of *M. bovis* and *M. caprae* in Spain

The objective of this chapter was to perform large-scale studies of *M. bovis* and *M. caprae* isolates from Spain by spoligotyping. Although spoligotyping has been applied in Spain for the past 15 years, no countrywide study had been conducted since 1996 in order to assess the degree of diversity within the Spanish *M. bovis* and *M. caprae* population and to compare the Spanish situation with results from other European countries.

Two scientific papers derived from these studies:

- Rodríguez, S., B. Romero, J. Bezos, L. de Juan, J. Álvarez, E. Castellanos, N. Moya, F. Lozano, S. González, J. L. Sáez-Llorente, A. Mateos, L. Domínguez, and A. Aranaz, and the Spanish Network on Surveillance and Monitoring of Animal Tuberculosis. 2010.

High spoligotype diversity within a *Mycobacterium bovis* population: Clues to understanding the demography of the pathogen in Europe. Veterinary Microbiology. 141:89-95.

- Rodríguez, S., J. Bezos, B. Romero, de Juan L., J. Álvarez, E. Castellanos, N. Moya, F. Lozano, M. T. Javed, J. L. Sáez-Llorente, E. Liébana, A. Mateos, L. Domínguez, and A. Aranaz, and the Spanish Network on Surveillance and Monitoring of Animal Tuberculosis. 2011. ***Mycobacterium caprae* infection in livestock and wildlife, Spain.** Emerging Infectious Diseases 17:532-535.

Moreover, the following contributions to conferences and meetings of European projects were presented:

- Rodríguez, S., A. Aranaz, B. Romero, L. de Juan, J. Bezos, J. Álvarez, E. Castellanos, N. Moya, F. Lozano, A. Mateos and L. Domínguez. **Spoligotyping diversity of *Mycobacterium bovis* in Spain.** Oral presentation. Workshop "VNTR/MIRU and DVR-spoligotyping for *Mycobacterium bovis* typing" of European project SSPE-CT-2004-501903. Toledo (Spain), 19th-21st October 2006.

- Rodríguez, S., E. Castellanos, J. Bezos, A. Aranaz, L. de Juan, F. Lozano, A. Mateos and L. Domínguez.. **The usefulness of DVR-spoligotyping in characterizing Spanish isolates of the zoonotic agents *Mycobacterium bovis* and *Mycobacterium caprae*.** Poster. 3rd Med-Vet-Net Annual Scientific Meeting. Lucca (Italy), 27th-30th June 2007.

- Rodríguez, S., J. Bezos, L. de Juan, B. Romero, J. Álvarez, E. Castellanos, S. González, J. L. Sáez, A. Mateos, L. Domínguez and A. Aranaz. **Molecular epidemiology underlines the importance of *Mycobacterium caprae* in livestock and wildlife.** Oral presentation. 31st Annual Congress of the European Society of Mycobacteriology. Bled (Slovenia), 4th-7th June 2010.

In addition, further studies were conducted that are included thematically in this chapter:

- The global distribution of spoligotypes. Review of spoligotype patterns described in the bibliography on *M. bovis* and *M. caprae* isolates and included in the database www.mbovis.org.

- Dendrograms of the spoligotypes present in Spain.

Chapter II: Molecular typing as a tool in tracking outbreaks caused by *M. bovis*

The objective of this chapter was the evaluation of the combination of spoligotyping with the VNTR typing technique in several selected sets of isolates of *M. bovis*. In the last years VNTR typing has become an important molecular tool for the

tracking of tuberculosis outbreaks. Different studies using VNTR typing report that the specific geographical setting has to be taken into account to define a set of markers able to achieve the best results. In this chapter the allelic diversity of several markers and the overall discriminatory power of VNTR typing was assessed in different strains of *M. bovis* in order to determine a suitable combination of loci for typing Spanish isolates.

The studies comprised herein resulted in a scientific paper and a manuscript:

- Rodriguez-Campos, S., A. Aranaz, de Juan L., J. L. Sáez-Llorente, B. Romero, J. Bezos, A. Jiménez, A. Mateos, and L. Domínguez. 2011. **Limitations of Spoligotyping and Variable Number Tandem Repeat Typing for Molecular Tracing of *Mycobacterium bovis* in a High Diversity Setting.** Journal of Clinical Microbiology 49:3361-3364.

- Rodriguez-Campos, S., B. Romero, L. de Juan, J. Bezos, A. Mateos, L. Domínguez and A. Aranaz. **Discrimination of variable number repeat typing rises with the expansion of a clonal group of *Mycobacterium bovis*.** Manuscript in preparation.

Furthermore, the following collaboration in a scientific paper arose from these studies:

- García-Bocanegra, I., I. Barranco, I. M. Rodríguez-Gómez, B. Pérez, J. Gómez-Laguna, S. Rodríguez, E. Ruiz-Villamayor and A. Perea. 2010. **Tuberculosis in alpacas (*Lama pacos*) caused by *Mycobacterium bovis*.** Journal of Clinical Microbiology. 48:1960-1964.

The results were presented at the following conferences and meetings of European projects:

- Rodríguez, S., E. Castellanos, L. de Juan, J. Bezos, F. Gallardo, N. Moya, J. Álvarez, N. Álvarez, T. Alende, A. Gutiérrez, F. Lozano, A. Mateos and B. Romero. **Mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) typing of SB0121, the most frequent spoligotype in Spain.** Oral presentation. Workshop "VNTR/MIRU and DVR-spoligotyping for Mycobacterium bovis typing. Results of the VNTR-DVR Spoligotyping Ring Trial". of European project SSPE-CT-2004-501903. Madrid (Spain), 24th-25th March 2009.

- Rodríguez, S., A. Aranaz, J. Bezos, E. Castellanos, L. de Juan, F. Gallardo, A. Gutiérrez, A. Mateos, L. Domínguez. and B. Romero. **High discrimination of the MIRU-VNTR technique for the most frequent spoligotype in Spain.** Oral presentation. M. bovis V Conference. Wellington (New Zealand), 25th-28th August 2009.

- Rodríguez, S., **Advances in Workpackage 6, Partner 1: Molecular characterisation of *M. bovis* and *M. caprae* isolates focused on epidemiological investigation.** Mid-term meeting of European project FP7-KBBE-2007-212414. Madrid (Spain). 11th-12th November 2010.

Moreover, another study is included thematically in this chapter:

- Analysis of a set of strains of *M. bovis* originating from bullfighting cattle by spoligotyping and VNTR typing. Manuscript in preparation.

Chapter III: The Spanish national database of animal tuberculosis - mycoDB.es

Chapter III comprehends a direct contribution to the Spanish national eradication programme of bovine tuberculosis - the national database of animal tuberculosis "mycoDB". With spoligotyping becoming a standard technique within the national campaign, centralisation of the typing data became indispensable and a database was designed and set up in collaboration with the Spanish Ministry of the Environment, Rural and Marine Affairs (MARM) to fulfil this need. Later on the database was extended to harbour VNTR typing data as well. Access to the data is granted by the MARM to Official Veterinary Services and Regional Laboratories. Knowledge of the intra- and interspecies and geographic dissemination of the different spoligotypes is required to elucidate relationships between farms, e.g. by animal movement, and between livestock and wildlife. Furthermore, the possibility of the national authorities involved in the eradication of bovine tuberculosis to obtain molecular data at a glance, improves the collaboration with Public Health institutions.

The following scientific paper resulted from this work:

- Rodriguez-Campos, S., S. González, L. de Juan, B. Romero, J. Bezos, C. Casal, J. Álvarez, I. G. Fernández-de-Mera, E. Castellanos, A. Mateos, J. L. Sáez-Llorente, L. Domínguez, and A. Aranaz, and The Spanish Network on Surveillance and Monitoring of Animal Tuberculosis. 2011. **A database for animal tuberculosis (mycoDB.es) within the context of the Spanish national programme for eradication of bovine tuberculosis.** Infection, Genetics and Evolution. In press.

Moreover, the following contributions to conferences and meetings of European projects were presented:

- Rodríguez, S. **The national database of *Mycobacterium bovis* and *Mycobacterium caprae*. Use in epidemiological surveys.** Oral presentation. Meeting of the bovine tuberculosis subgroup of the Task Force (EFSA). Seville (Spain), 14th-15th November 2007.

- Rodríguez, S., B. Romero, L. de Juan, S. González, J. Bezos, J. Álvarez, E. Castellanos, F. Lozano, N. Moya, N. Álvarez, T. Alende, A. Gutiérrez, F. Gallardo, A. Mateos, A. Aranaz and L. Domínguez. **The national database of Spanish *Mycobacterium bovis* and *Mycobacterium caprae* isolates.** Oral presentation. Workshop "VNTR/MIRU and DVR-spoligotyping for *Mycobacterium bovis* typing. Results of the VNTR-DVR Spoligotyping

Ring Trial". of European project SSPE-CT-2004-501903. Madrid (Spain), 24th-25th March 2009.

- Romero, B., S. Rodríguez, J. Bezos, J. Álvarez, E. Castellanos, S. González, F. Lozano, N. Moya, A. Gutiérrez, T. Alende, J. L. Sáez, A. Mateos, A. Aranaz and L. Domínguez. **Spanish Database of animal mycobacteriosis.** Poster. Final meeting European project SSPE-CT-2004-501903. Turin (Italy), 17th-19th June 2009.

- de Juan, L., S. Rodríguez, B. Romero, A. Aranaz, J. Bezos, E. Castellanos, S. González, J. L. Sáez, Ana Mateos and L. Domínguez. **Spanish database of animal mycobacteriosis (mycoDB): application in epidemiological studies.** Oral presentation. 31st Annual Congress of the European Society of Mycobacteriology. Bled (Slovenia), 4th-7th June 2010.

Chapter IV: Phylogeny of *M. bovis* in the Iberian Peninsula

While the global phylogeny of *M. tuberculosis* is disclosed in several studies regarding *M. tuberculosis* lineages and their geographic distribution, the phylogeny of *M. bovis* was only poorly understood until recently. The objective of this chapter was the study of Spanish isolates in relation to two clonal complexes of *M. bovis* and the analysis of the most common ancestor (MRCA) of the Spanish *M. bovis* population. The study of the MRCA aimed at the identification of a phylogenetic marker for Spanish *M. bovis* isolates and the assessment of its presence in Europe. Since the Spanish and Portuguese *M. bovis* populations are closely related, this study was extended to the Iberian Peninsula.

A scientific paper derived from this study:

- Rodriguez-Campos, S., A. C. Schürch, J. Dale, A. J. Lohan, M. V. Cunha, A. Botelho, K. De Cruz, M. L. Boschiroli, M. B. Boniotti, M. Pacciarini, M. C. Garcia-Pelayo, B. Romero, L. de Juan, L. Domínguez, S. V. Gordon, D. van Soolingen, B. Loftus, S. Berg, R. G. Hewinson, A. Aranaz and N. H. Smith. **European 2 – a clonal complex of *Mycobacterium bovis* dominant in the Iberian Peninsula.** Infection, Genetics and Evolution. In press.

Collaboration in two scientific papers arose from this study:

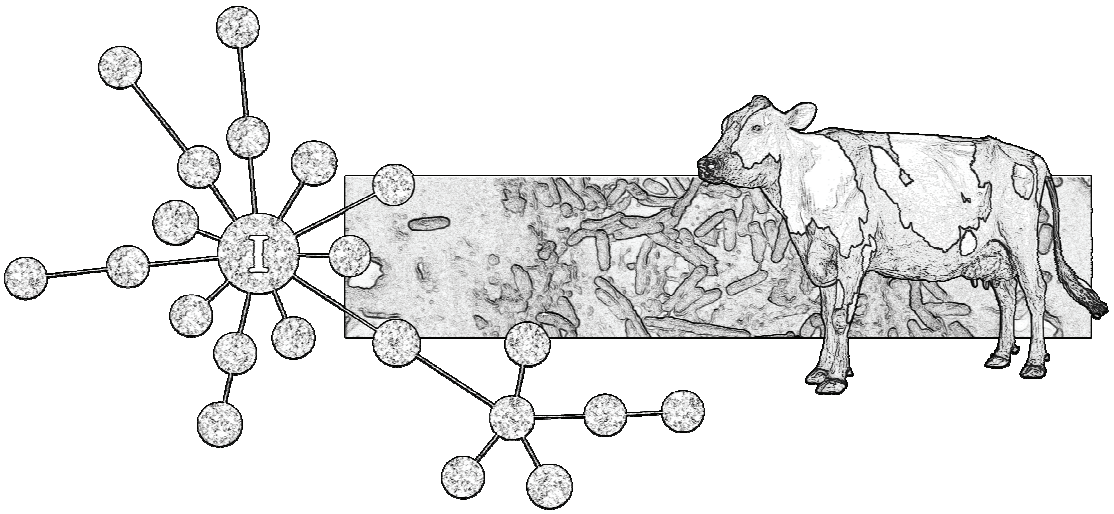
- Berg, S., M. C. Garcia-Pelayo, B. Müller, E. Hailu, B. Asiimwe, K. Kremer, J. Dale, M. B. Boniotti, S. Rodríguez, M. Hilty, L. Rigouts, R. Firdessa, A. Machado, C. Mucavele, B. Nare Ngandolo, J. Bruchfeld, L. Boschiroli, A. Müller, N. Sahraoui, M. Pacciarini, S. Cadmus, M. Joloba, D. van Soolingen, A. L. Michel, B. Djønne, A. Aranaz, J. Zinsstag, P. van Helden, F. Portaels, R. Kazwala, G. Källenius, R. G. Hewinson, A. Aseffa, S. V. Gordon and N. H. Smith. 2011. **African 2, a clonal complex of *Mycobacterium bovis* epidemiologically important in East Africa.** Journal of Bacteriology 193:670-678.

- Smith, N. H., S. Berg, J. Dale, A. Allen, S. Rodríguez, B. Romero, F. Matos, S. Ghebremichael, C. Karoui, C. Donati, A. da Conceicao Machado, C. Mucavele, R. R. Kazwala, M. Hilty, S. Cadmus, B. N. R. Ngandolo , M. Habtamu, J. Oloya, A. Müller, F. Milian-Suazo, O. Andrievskaia, M. Projahn, S. Barandiarán, A. Macías, B. Müller, M. Santos Zanini, C. Y. Ikuta, C. A. Rosales Rodriguez, S. R. Pinheiro, A. Figueroa, S. N. Cho, N. Mosavari, P. N.Chuang, J. Zinsstag, D. van Soolingen, E. Costello, A. Aseffa, F. Proaño-Perez, F. Portaels, L. Rigouts, A. A.Cataldi, D. M. Collins, M. L. Boschiroli, R. G. Hewinson, J. S. Ferreira Neto, Om Surujballi, K. Tadyon, A. Botelho, A. M. Zárraga, N. Buller, R. Skuce, R. Jou, A. Michel, A. Aranaz, B.-Y. Jeon, G. Källenius, S. Niemann, M. B. Boniotti, P. D. van Helden, B. Harris, M. J. Zumárraga and K. Kremer. 2011. **European 1: A globally important clonal complex of *Mycobacterium bovis***. Infection, Genetics and Evolution 11:1340-1351.

Altogether the four chapters of this PhD thesis include eight published papers, three of them resulting from collaborations with different institutions. Moreover, a manuscript ready for submission is included: "Discrimination of variable number repeat typing rises with the expansion of a clonal group of *Mycobacterium bovis*". A further study using spoligotyping and variable number repeat typing in a selection of isolates from bullfighting cattle is resumed in chapter II and the corresponding manuscript is in preparation. Part of the introduction of this thesis will be submitted as review with the title: "An update of molecular typing of *Mycobacterium bovis* and *Mycobacterium caprae* and its relevance for epidemiological studies".

Chapter I

Molecular demography of *Mycobacterium bovis* and *Mycobacterium caprae* in Spain



Molecular demography of *M. bovis* and *M. caprae* in Spain

Mycobacterium (*M.*) *bovis* and *M. caprae* are the main causative agents of animal tuberculosis in Spain and have been isolated from several domestic and wild animal species. The spoligotyping technique (Kamerbeek *et al.*, 1997) has been extensively used to type Spanish strains (Aranaz *et al.*, 1996; Gutiérrez *et al.*, 1997; Parra *et al.*, 2003; Gortázar *et al.*, 2005) and the results pointed at a higher diversity of spoligotypes than that observed in population studies from other countries. To evaluate the epidemiological situation regarding the distribution of *M. bovis* and *M. caprae* isolates in Spain in the different Autonomous Communities, geographical regions and infected animal species, two large population surveys were conducted including spoligotyping results from 6215 *M. bovis* (years 1992-2007) and 791 *M. caprae* (years 1992-2009) isolates.

M. bovis was found all over the national territory, excluding the Autonomous Communities Murcia, Valencia, Ceuta and Melilla, and infected three domestic animal species (cattle, goat and swine), seven wildlife species (wild boar, red deer, fallow deer, Iberian lynx, fox, chamois and badger), pets (cat and dog) and zoo animals (mouflon). Spoligotyping resulted in 252 spoligotype patterns yielding a discriminatory index (Hunter and Gaston, 1988; Hunter, 1990) of 0.87. Such spoligotype diversity is unusual in other countries including Great Britain (Hewinson *et al.*, 2006), Northern Ireland (Skuce *et al.*, 2005) and Australia (Cousins *et al.*, 1998). Regarding mainland Europe, similarities of spoligotype patterns and diversity exist between Spain, France, Portugal and Italy (Haddad *et al.*, 2001; Duarte *et al.*, 2008; Boniotti *et al.*, 2009). For example, SB0121, the most frequent spoligotype in Spain (27.9%), is also the most abundant spoligotype in Portugal and is present, although at lower frequencies, in France and Italy. Spoligotype SB0121 differs from the BCG-like spoligotype, which is the most frequent type in France and Italy, in the absence of spacer 21. The loss of spacer 21 is a dominant feature among Spanish isolates with 67.1% of them displaying spoligotypes that lack this spacer. The reasons for the abundance of SB0121 strains are not yet disclosed; on the one hand, strains with this spoligotype pattern could have a selective advantage over other strains, on the other hand, it could be that the spoligotyping technique lacks discriminatory power to further distinguish these strains.

Considering the different animal species the biggest variety of spoligotypes was found in cattle, from which 239 patterns were obtained; out of these patterns 207 were exclusively found in this animal species. Generally, the most frequent spoligotypes were shared between domestic animals and wildlife and were found throughout the country; exceptions could be linked to certain regions where intensive sampling for typing purposes had been carried out in short time periods.

M. caprae, the main cause of caprine tuberculosis, can be identified based on the absence of spacers 1, 3 to 16, 28 and 39 to 43 from the spoligotype pattern; notwithstanding, additional molecular identification is advisable and was carried out in 63 isolates selected from the total of 791 isolates by three-primer PCR as per Mostowy *et al.* (2002). Moreover, one isolate of each spoligotype pattern was studied by sequencing the complete *pncA* gene and part of the *gyrB* gene to determine the specific *M. caprae* gene polymorphisms. Further unpublished data were obtained by sequencing of the 5' fragment of the DR region of five isolates of the three most prevalent *M. caprae* spoligotypes in Spain [SB0157 (n=1), SB0416 (n=3), and SB1084 (n=1)]. The region (accession no. Z48304) was amplified with forward primer DR681 (nucleotides 681 to 701, upstream the first DVR) and reverse primer DR2525 (2506 to 2525, located at the IS6110 element inserted in the locus). The analysis of this region showed a high level of homogeneity, as all five isolates investigated shared the deletions of DVR1 and DVR2, and DVR4 to DVR26 [following numbering of van Embden (2000)] that include the spacers 1, and 3 to 16 detected in the spoligotyping membrane in use. These deletions comprised exactly the DR and the subsequent spacer. DVRs 27, 28, and 30 (spacers 17, 18 and 20) were present in the sequenced isolates, and variable results were obtained with DVR29 (spacer 19) and DVR31 to 34 (spacers 21 to 24). Interestingly, except in spoligotype patterns SB0418 and SB1619 had spacers 30 to 33 present, while these were consistently absent from the other strains; both spoligotypes had been isolated from imported cattle of eastern European origin.

The strain diversity found among the *M. caprae* isolates was low compared to *M. bovis*; 791 *M. caprae* isolates clustered in 15 spoligotype patterns (discriminatory index D=0.58). Most of the isolates originated from goats (68.5%), but were also obtained from a considerable number of cattle (28.9%) and also from sheep, domestic pig, wild boar, red deer and fox. The importance of this pathogen in cattle is reflected by a statistically significant increase of *M. caprae* infections since 2004. Although more infections with *M. caprae* were observed in cattle in regions with a high goat density, the fact that most of the affected farms did not have contact to small ruminants highlights the maintenance of this pathogen within the cattle population.

Additionally, variable number tandem repeat (VNTR) typing with eight loci (ETR-A, ETR-B, ETR-D, QUB11a, QUB11b, QUB3232, ETR-E, and MIRU26) was performed on a selection of 20 isolates that originated from 10 properties, each displaying 2 different spoligotypes at a time. At five farms the change of the spoligotype pattern could be explained by a single deletion event while the VNTR profile remained identical.

For all spoligotype patterns the international nomenclature was obtained on www.mbovis.org (Smith and Upton, 2011). A review of spoligotype patterns with *M.*

bovis-, *M. caprae*-, *M. microti*- or *M. pinnipedii*-like spoligotyping patterns from www.mbovis.org (consulted on 29 November 2011) amd published in large population surveys can be found in Appendix VI (provided in electronic format).

Spoligotyping profiles have often been compared using hierarchical clustering methods such as unweighted pair group method with arithmetic mean (UPGMA) in order to visualise relationships between the different patterns. We used the UPGMA method in three different selections of the Spanish spoligotypes: 1) *M. bovis* spoligotypes with spacer 21 present in the spoligotype pattern, 2) *M. bovis* spoligotypes with spacer 21 deleted from the spoligotype pattern, and 3) spoligotypes belonging to *M. caprae* strains.

Authorisation of the co-authors was granted to include the following articles in the thesis and necessary permissions from the journals were obtained for reproducing them in the printed thesis and its online version. Extensive supplementary data are not shown, but the link to the corresponding website is provided.

I.1. High spoligotype diversity within a *M. bovis* population

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High spoligotype diversity within a *Mycobacterium bovis* population: Clues to understanding the demography of the pathogen in Europe

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ABSTRACT

Mycobacterium bovis is the main causative agent of bovine tuberculosis. This zoonotic disease produces important economic losses and must be considered a threat to endangered animal species and public health. This study was performed (1) to assess the degree of diversity of the Spanish *M. bovis* isolates and its effect on the epidemiology of the infection, and (2) to understand the connection of *M. bovis* populations within a European context. In this report we resume the DVR-spoligotyping results of 6215 *M. bovis* isolates collected between 1992 and 2007 from different hosts. The isolates clustered into 252 spoligotypes which varied largely in frequency, geographical distribution and appearance in different animal species. In general, the most frequent spoligotypes were found all over the country and in different animal species, though some were restricted to a geographical area. Among our most often isolated spoligotypes, SB0121 and SB0120 (BCG-like) are a common feature between mainland European countries, however, the spoligotypes differ with those found in the UK, the Republic of Ireland and abroad. A comparison of spoligotypes reported from other countries reveals hints for the *M. bovis* demography in Europe and suggests a common ancestor strain. This study gives insight into the usefulness of the standardized DVR-spoligotyping technique for epidemiological studies in a country with a high degree of strain diversity.

1. Introduction

Mycobacterium bovis, the main causative agent of bovine tuberculosis, affects a wide range of domestic animals and wildlife. This infection entails important environmental, economic and public health risks (Briones et al., 2000; Corner, 2006; Michel et al., 2006).

In Spain all regions are subjected to a national eradication programme based on intradermal tuberculin testing of cattle and the slaughter of reactor animals. The annual herd period prevalence rate has been reduced to 1.63% in 2007. However, large differences exist between geographical areas (0.19–9.51%) (MARM, 2007).

Nowadays we count on a set of molecular typing techniques in order to investigate epidemiological backgrounds. Direct variable repeat spacer oligonucleotide

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typing (DVR-spoligotyping) (Kamerbeek et al., 1997) detects polymorphism within the genomic direct repeat (DR) locus. The DR locus consists of multiple sequences interspersed with non-repetitive spacer sequences (spacers). Strains vary in the presence and absence of these spacers and can therefore be clustered in so-called spoligotypes. Since the implementation of spoligotyping for *M. bovis* (Aranaz et al., 1998; Roring et al., 1998), the technique has been considered useful as a fast and cost-effective method for first-line typing (Haddad et al., 2004; Milian-Suazo et al., 2008). The applicability of the technique can vary between countries or even regions, because of the differences between strain diversity and the discriminatory power of spoligotyping. However, the reasons underlying these differences in diversity remain largely unknown.

This paper provides a survey of the *M. bovis* population in Spain by resuming the spoligotyping results for 6215 isolates obtained during the last 15 years. We describe the spoligotype diversity of *M. bovis* and offer insight into the usefulness of spoligotyping for epidemiological studies when a high degree of strain diversity is present.

2. Materials and methods

2.1. Mycobacterial isolates and strains

This study comprises 6215 *M. bovis* isolates from Spain, collected between January 1992 and December 2007. Most of the isolates were cultured in the laboratory VISAVET (Facultad de Veterinaria, Universidad Complutense Madrid), but about 40% of the isolates were submitted by Regional Laboratories within the national bovine tuberculosis eradication program. The isolates were obtained from cattle (*Bos taurus*, $n = 5585$), domestic goats (*Capra aegagrus hircus*, $n = 33$), domestic pigs (*Sus scrofa domestica*, $n = 7$), wild boars (*Sus scrofa*, $n = 204$), red deer (*Cervus elaphus*, $n = 141$), fallow deer (*Dama dama*, $n = 229$), Iberian lynxes (*Lynx pardinus*, $n = 6$), foxes (*Vulpes vulpes*, $n = 2$), chamois (*Rupicapra rupicapra*, $n = 2$), a badger (*Meles meles*, $n = 1$), cats (*Felis silvestris catus*, $n = 3$), a dog (*Canis lupus familiaris*, $n = 1$) and a mouflon (*Ovis musimon*, $n = 1$, zoo animal). The geographical distribution is represented in the supplementary figure according to the number of isolates.

The tissue samples were decontaminated and cultured onto Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein–Jensen media (bioMérieux España and Biomedics, Madrid, Spain) and incubated at 37 °C. The DNA was prepared from colonies by suspending them in 200 μl of distilled water and boiling for 10 min at 100 °C. The isolates were confirmed as members of the *M. tuberculosis* complex by acid–alcohol–fast staining and PCR amplification of *Mycobacterium* genus-specific 16S rRNA fragment and MPB70 sequences (Wilton and Cousins, 1992).

2.2. DVR-spoligotyping

The isolates were spoligotyped following the protocol described by Kamerbeek et al. (1997). The DR region was amplified using the primers DRa (GGTTTTGGGTCTGACGAC, 5′ biotinylated) and DRb (CCGAGAGGGGACGGAAAC) (Roche Molecular Biochemical, Berlin, Germany) and the amplified product was hybridised onto a spoligotyping membrane (Isogen Bioscience BV, Maarssen, The Netherlands). The DNA was detected with the streptavidin–peroxidase conjugate (Boehringer-Mannheim, Germany) and the ECL system (GE Healthcare, Barcelona, Spain), followed by exposure of an X-ray film to the membrane. We included purified sterile water as a negative control and a clinical isolate of *M. tuberculosis* (Aranaz et al., 1996) as a positive control in every PCR-batch and hybridisation assay. Authoritative names (prefix SB followed by four digits) for spoligotype patterns were obtained from the *M. bovis* Spoligotype Database website (http://www.Mbovis.org). Part of the results have been previously published [5% (Aranaz et al., 2004) and 1.6% (Romero et al., 2008)].

The spoligotyping results were enlisted in a Microsoft® Office Access database along with the epidemiological data (isolation date, animal species and geographical origin).

2.3. Discriminatory power calculation

The index of discrimination (D) described by Hunter and Gaston (1988) was calculated in order to determine the discriminatory power of the spoligotyping technique at the national level and for provinces (Spanish administrative subdivision). We used the in silico website of the University of the Basque Country (http://www.insilico.ehu.es), filling in the number of unrelated strains for each spoligotype. For this purpose we only counted one spoligotype when isolates of the same herd or a precise geographical area (i.e. National Parks, hunting estates) shared identical patterns.

Additionally, we calculated the quotient of the number of unrelated strains (with unknown epidemiological link) and the number of different spoligotyping patterns observed for that region in order to describe the degree of diversity in each province.

3. Results

3.1. Outline

The 6215 *M. bovis* isolates included in this study originated from sampling performed during 1992–2007 that almost achieved full country coverage, excluding only the Autonomous Regions Murcia, Valencia, Ceuta and Melilla (supplementary Figure). The characterization by spoligotyping of the 6215 *M. bovis* isolates resulted in 252 different patterns (supplementary Table).

The 15 most frequent Spanish types (the ones which made up at least 1% of our samples) represent 77.43% of the totality of the isolates; the corresponding prevalence rates are shown in Table 1. Interestingly, 12 of the 15 most frequent types appeared throughout the years covered by this study, always accounting for approximately the same fraction of the total strains. The other three types (SB0135, SB1232 and SB1258) were associated with intensive sampling of domestic and/or wildlife animals in determined areas. The most frequent spoligotype is SB0121 [spb-7 in previous reports (Aranaz et al., 1996)] that

Table 1
Distribution of the spoligotypes of the Spanish *Mycobacterium bovis* isolates according to prevalence [frequent types (>1%), infrequent types and orphans (singular types)] by animal species.

Spoligotype	No. of isolates from												
	Cattle	Goat	Pig	Deer	Wild boar	Iberian lynx	Fox	Dog	Cat	Mouflon	Badger	Chamois	Prevalence
SB0140	62				1								1.01
SB1305	65												1.05
SB1258	66					1		1	1				1.11
SB1337	90												1.45
SB0135	104			1	1								1.71
SB0152	106				1								1.72
SB0119	112			4	13								2.08
SB1232	37			49	54	3	1						2.32
SB0130	204			2	4				1				3.40
SB0120	241			2	3								3.96
SB0295	240	1	1	3	8	2							4.10
SB0265	377		6	2	7								6.31
SB0339	235			254	12								8.06
SB0134	686			2	9								11.21
SB0121	1653	31		16	37								27.94
Orphans (84)	76	1		3	2				1	1			1.35
Others (153)	1231			32	52		1				1	2	21.22

clustered 27.94% of the isolates, followed by SB0134 (spb-13) and SB0339 (spb-16) which accounted for 11.21 and 8.06% of the isolates (Table 1).

The 237 other spoligotyping patterns comprised 153 patterns (1319 isolates) with very low frequency (28.1% of them had been found infecting only one herd each), and 84 singular patterns, so-called orphans, which were only isolated once (Table 1).

Altogether we identified 148 spoligotypes that have not been previously listed on www.Mbovis.org. The 252 spoligotypes are attached as supplementary table.

3.2. Discriminatory power and diversity ratio

We calculated the discriminatory power (D) for the spoligotyping of the Spanish *M. bovis* isolates, which means the average probability that the technique will assign a different type to two isolates randomly sampled in the population of *M. bovis*, using the equation by Hunter and Gaston (Hunter and Gaston, 1988; Hunter, 1990). We achieved a discriminatory index $D = 0.87$ at national level.

We also calculated the discriminatory index of spoligotyping in Spanish provinces from which more than 60 isolates were obtained, and D oscillated between 0.74 and 0.93. In addition, the strain/pattern ratio ranged from 1.5 to 7.1 (supplementary Figure). We compared these results with the respective number of isolates and the annual herd period prevalence of bovine tuberculosis (MARM, 2007). In general, provinces with lower herd prevalence (<0.8%) showed a slightly higher D value (average $D = 0.90$) compared to national average and lower strain/pattern ratio (average ratio = 2.7), whereas provinces with extreme herd prevalence (>4%) showed lower D values (average $D = 0.82$) and higher strain/pattern ratio (average ratio = 4).

3.3. Geographical distribution

The *M. bovis* strains were unequally distributed all over Spain (supplementary Table). The predominant patterns were repeatedly found in all regions and in different animal species. We observed accumulation of some spoligotypes in certain regions. For instance, SB0339 was isolated from animals from central and northern regions, whereas SB1230 originated from isolates from the South. The analysis of the geographical distribution of 137 types (excluding the orphan and epidemiologically linked isolates) showed that the majority (75.9%) is disseminated while 33 types appear localized. The strains that appeared in delimited areas and presented a high prevalence, especially SB0339 (Monte El Pardo Nature Reserve) and SB1232 (Doñana National Park), must not be overestimated, given that intensive sampling for study purposes had been carried out in those areas.

3.4. Animal species

The vast majority of the types were isolated from cattle, in fact 207 of our spoligotypes were found only in this species (Table 2). Six patterns were exclusively isolated from wild boars (SB0868, SB1095, SB1260, SB1274,

Table 2
Distribution of the *Mycobacterium bovis* isolates according to animal species, and number of spoligotypes which were found only in a determined species.

Animal species	Isolates	Types	Exclusive types
Cattle	5585	239	207
Wild boar	204	26	6
Red deer	141	22	2
Goat	33	3	1
Cat	3	3	1
Mouflon	1	1	1
Fallow deer	229	13	1
Pig	7	2	0
Iberian lynx	6	3	0
Chamois	2	1	0
Fox	2	2	0
Badger	1	1	0
Dog	1	1	0

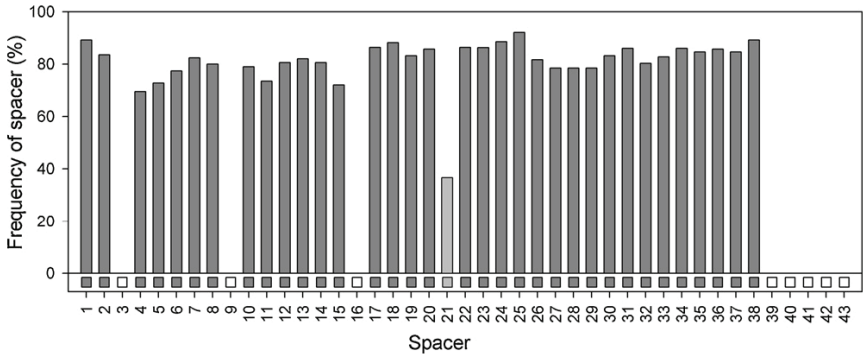

Fig. 1. Frequencies of the 43 spacers of the DR locus that are included in the spoligotyping membrane (designed by Kamerbeek et al., 1997) detected by PCR and reverse-blotting. Data expressed as percentage of spoligotypes ($n = 252$) in which the spacer is present.

SB1336, and SB1367). Another five singular patterns were obtained from samples of red deer (SB1330 and SB1393), a goat (SB1062), a mouflon (SB1280) and a cat (SB0972). The cattle-specific spoligotypes represented 86.61% of the patterns found in *M. bovis* from cattle. By comparison, the percentage of exclusive types from wild boar and red deer was notably lower (23.08 and 9.09%, respectively).

Interestingly, the most common spoligotypes were shared by different animal species, as shown in Table 1. There are only two exceptions: SB1305, isolated from cattle from the north of Spain, and SB1337 isolated from cattle from Catalonia. In both regions intensive sampling for typing purposes has been carried out in 2006 and 2007.

3.5. Frequency of spacers

We compared the frequencies of the 43 spacers throughout the 252 spoligotypes (Fig. 1) and could confirm the absence of spacers 3, 9, 16, and 39–43. Though none of the spacers were present in all the patterns, we found a rather homogenous distribution. Each spacer was present in about 79.96% (69.5–89.2%) of the strains, except for spacer 21 which could be found in only 36% of our strains. The patterns which lack this spacer account for 67.13% of the typed isolates, but neither belonged to a determined animal species, nor geographical area.

4. Discussion

In this study we report the characterisation of a representative collection of *M. bovis* isolates from the different animal host species described in Spain. This collection resulted from the involvement of numerous contributors which reflects the commitment at national level on the study of the epidemiology of bovine tuberculosis. The DVR-spoligotyping clustered the isolates in 252 spoligotypes with a discriminatory index of $D = 0.87$. To our knowledge this study reveals the highest diversity within a *M. bovis* population that has been described in scientific literature.

While most spoligotypes were distributed throughout the country, a small number of patterns were restricted to determined regions. Consequently, extension of the analysis to Spanish regions not studied before yields patterns not previously described. In general, we found a higher discriminatory index for spoligotyping in the Spanish northern regions which started eradication of bovine tuberculosis first and present lower annual herd period prevalence. However, some exceptions exist, likely reflecting animal species, cattle breeds and herd management. This fact suggests that test-and-slaughter policy has not affected the local strain diversity.

Spoligotyping results show common traits among western continental EU countries and to a limited extent also to the British Isles. As previously described in other reports, a frequent type clusters a high percentage of isolates. Our most often identified spoligotype, SB0121, is also the most frequent strain in Portugal (Duarte et al., 2008), the second most predominant type in France after SB0120 (Haddad et al., 2001) and one of the five most frequent Italian types (Boniotti et al., 2009). The similarities between Spain, France, Portugal and Italy can be due to the geographical nearness and trade relationship. SB0121 has also been found with a very low frequency (<1%) in mainland Great Britain (Hewinson et al., 2006). Surprisingly, SB0120 represents 3.95% of the isolates in Spain but is infrequent in Portugal (Duarte et al., 2008). Spoligotypes SB0130 and SB0134, which make up 3.39 and 11.19% of our isolates, respectively, were isolated frequently in Britain (Smith et al., 2006) and Ireland (only SB0130) (Costello et al., 1999; Skuce et al., 2005). SB0134 was also reported from French cattle and wildlife (Zanella et al., 2008). Furthermore, we have repeatedly isolated SB0140, the most common spoligotype of the British Isles. Advanced studies using additional techniques such as Variable Number Tandem Repeat analysis would be needed to reveal further epidemiological relationships.

The analysis of diversity within each species offered insight into the relative contribution of livestock and wildlife to the epidemiology of bovine tuberculosis in Spain. Wildlife, especially wild boar has been suggested as a reservoir (Aranaz et al., 2004; Hermoso de Mendoza et al., 2006; Naranjo et al., 2008). The finding of 207 spoligotypes exclusive to cattle gives evidence that cattle-specific spoligotypes are responsible for much of the diversity of the Spanish *M. bovis* population. The involvement of

wildlife in the epidemiology of the infection is highlighted by the fact that 12 out of the 15 most frequent types are present both in cattle and at least in one wild *Artiodactyla* species. The special farming system in Spain may have promoted the transmission from cattle to sympatric wildlife and the broad host range of these strains reflects its success as a pathogen.

The results of this study also contribute to the knowledge of the demography of *M. bovis* in Spain within the European context. A comparison of the spoligotypes and *D* values reported in publications could explain the spread of *M. bovis* infection. Modern genetic and archeological evidence suggests that the domestication centre of European cattle (*B. taurus*) was the Near East at the very beginning of the Neolithic period (Beja-Pereira et al., 2006; Edwards et al., 2007). Recent articles of cattle phylogeny based on mitochondrial DNA showed the origin of European cattle in the Fertile Crescent and the subsequent expansion from this ancestral population (Götherström et al., 2005; Beja-Pereira et al., 2006). They also highlight the strong influence of cattle of North African origin introduced by maritime routes into the Mediterranean countries. These findings are coherent with the hypothesis of ancient cattle being infected with ancestral *M. bovis* strains which have a maximum number of DVR spacers. This ancestor would derive from a *M. tuberculosis*-like organism (Brosch et al., 2002; Mostowy et al., 2005; Smith et al., 2006). We assume that original cattle populations remained with human settlements, giving rise to locally adapted strains and further diversification. This evolutionary scenario is congruent with those described for the demography of the *M. tuberculosis* complex and its association with the human host (Wirth et al., 2008; Hershberg et al., 2008). Cattle arrived into Spain through the Pyrenees and along the Mediterranean cost during the Neolithic period. Likely, the cattle population increased in the region providing an ecological niche for clonal expansion of the founder strains. Due to the orography and history of the Iberian Peninsula, the exchange of cattle between certain regions has been impeded for a long time and therefore favoured an independent evolution of *M. bovis* strains. The high diversity in Spain may have been caused by this dual entrance of cattle from land and maritime routes. Nevertheless, we could not distinguish two specific spoligotyping signatures suggesting two major lineages that had evolved independently.

The evolution of the DR region is probably unidirectional, occurring by single spacer deletions or loss of contiguous spacer sequences (Fang et al., 1998; van Embden et al., 2000). Among our strains, SB0120 (BCG-like) is the most likely common ancestor, from which SB0121 may have emerged by loss of spacer 21. The role of spacer 21 and the possible implications of its deletion have to be elucidated, as it has also been described for Portuguese isolates.

5. Conclusion

DVR-spoligotyping revealed a high degree of strain diversity among Spanish *M. bovis* isolates ($D = 0.87$) and thus can be considered a useful tool for the study of epidemiology of the infection in our country. The distribution of spoligotypes was unequal in Spain both in terms of geographical presence and frequency, and even in infection in the different host species. The implementation of the international database (www.Mbovis.org) has simplified the comparison between countries. A comparison of spoligotypes available in the scientific literature reveals a hypothesis about the spread of bovine tuberculosis that has shaped the *M. bovis* population structure in Europe. Our next target is the completion of a national database which integrates conventional epidemiology and GIS in order to support the national eradication campaign by exploiting the molecular epidemiology data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2009.08.007.

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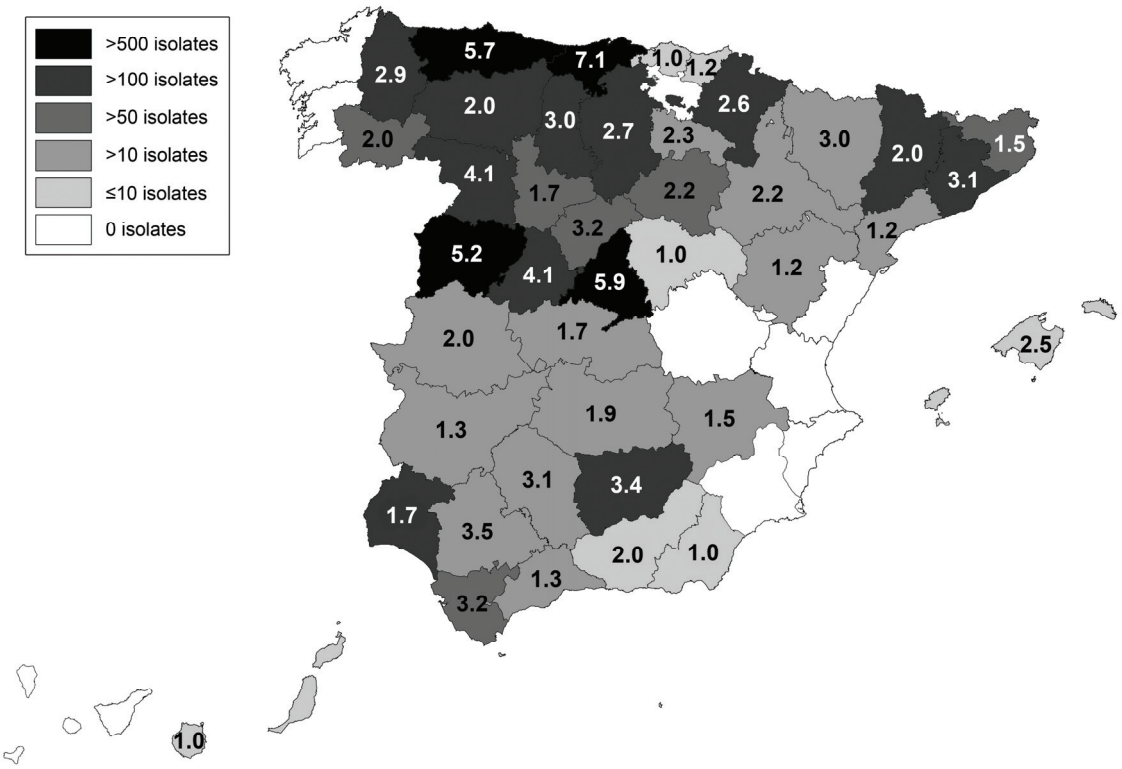

Supplementary figure. Map of Spain showing the geographical distribution of the *Mycobacterium bovis* isolates according to the number of isolates and the discriminatory indexes for the Spanish provinces from which more than 60 isolates were obtained.

Further supplementary material including all the spoligotype patterns described in this study, the respective frequency in Spain and their distribution by province can be found in the online version, at doi:10.1016/j.vetmic.2009.08.007.

I.2. *M. caprae* infection in livestock and wildlife

DISPATCHES

Mycobacterium caprae Infection in Livestock and Wildlife, Spain

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Mycobacterium caprae is a pathogen that can infect animals and humans. To better understand the epidemiology of *M. caprae*, we spoligotyped 791 animal isolates. Results suggest infection is widespread in Spain, affecting 6 domestic and wild animal species. The epidemiology is driven by infections in caprids, although the organism has emerged in cattle.

Mycobacterium caprae is a cluster within the *M. tuberculosis* complex (online Technical Appendix, www.cdc.gov/EID/content/17/3/532-Techapp.pdf). This pathogen has been recognized mainly in central Europe, where it has been occasionally isolated from tuberculous lesions from cattle (*1*–*5*), pigs (*4*), red deer (*Cervus elaphus*) (*4*,*5*), and wild boars (*Sus scrofa*) (*3*). Its isolation from humans has also been described (*3*,*6*); often, a contact with livestock has been suggested as a likely means of transmission (*5*). To our knowledge, this pathogen has never been isolated outside continental Europe, except from a European patient in Australia (*7*) and a cow in Algeria (*8*).

The combination of disease tracing and molecular typing is needed to understand the epidemiology of tuberculosis. This report describes the molecular epidemiology of *M. caprae* infection in Spain compared with other countries. We characterized *M. caprae* isolates from goats and other domestic and wild animals by spoligotyping (*9*). The relative contribution of each animal and its role in animal tuberculosis are discussed.

The Study

This study included 791 *M. caprae* isolates from domestic goats (*Capra aegagrus hircus*, n = 542), sheep (*Ovis aries*, n = 2), cattle (*Bos taurus*, n = 229), domestic pigs (*S. scrofa domestica*, n = 2), wild boars (*S. scrofa*, n = 14), red deer (*Cervus elaphus*, n = 1), and a fox (*Vulpes vulpes*, n = 1). The samples originated from skin test–positive animals identified within the national or regional eradication programs, from abattoir surveillance, and from postmortem inspections of wildlife, and were collected from 1992 through June 2009 in different geographic areas in Spain (Figure 1). Spoligotyping was performed as described (*9*), and authoritative names for spoligotype

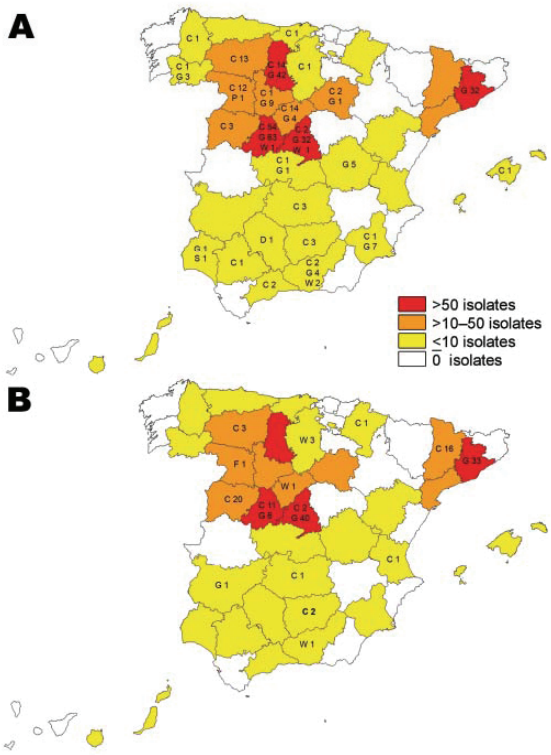

Figure 1. Map of Spain showing the distribution of the 2 most frequent *Mycobacterium caprae* spoligotypes and affected animals: C, cattle; D, red deer; F, fox; G, goats; S, sheep; P, pigs; WB, wild boar. A) Spoligotype SB0157. B) Spoligotype SB0416.

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[1]A list of members of The Spanish Network on Surveillance and Monitoring of Animal Tuberculosis can be found in the online Technical Appendix (www.cdc.gov/EID/content/17/3/532-Techapp.pdf).

patterns were obtained from the *Mycobacterium bovis* Spoligotype Database (www.mbovis.org).

Further authentication was achieved by detection of RD4 in the isolates with a 3-primer PCR in a panel of 63 unrelated isolates that included all spoligotyping patterns and animal species. Of the selected isolates, 62 showed the 545-bp product, indicating that they harbor RD4. One isolate from a cow of Eastern European origin repeatedly showed a 340-bp band, and its sequencing could not confirm presence or absence of RD4. For detection of specific *M. caprae* gene polymorphisms, 1 isolate from every spoligotyping pattern was studied. Additional identification was determined by sequencing of the pyrazinamidase A gene, which demonstrated a C at nt 169 that results in the functional wild-type pyrazinamidase A gene, and of the gyrase B gene that showed the G at nt 1311 and a C at position 1410 (online Technical Appendix).

The isolates, which originated from 195 single cases or outbreaks (Table 1), clustered into 15 patterns, which share the features previously described for the species (absence of spacers 1, 3–16, 28, and 39–43). Notably, the Iberian spoligotype cluster lacks spacers 30–33, whereas most *M. caprae* isolates from central Europe belong to spoligotypes that harbor these spacers. The 3 isolates of profiles SB0418 and SB1619 that presented spacers 30–33 originated from cattle imported from southeastern Europe. The 2 predominant spoligotypes, SB0157 and SB0416, were found to be responsible for 60% and 22%, respectively, of the cases and infected different animal species in distant areas, whereas 7 patterns were unique to a single case or outbreak. We calculated the index of discrimination (D) described by Hunter and Gaston (*10*) using the website of the University of the Basque Country (www.insilico.ehu.es). The result, D = 0.584, is notably lower compared with a parallel research of 252 patterns from 6,215 *M. bovis* isolates (D = 0.87) (*11*).

Additionally, variable number tandem repeat typing by using loci ETR-A, ETR-B, ETR-D, QUB11a, QUB11b, QUB3232, ETR-E, and MIRU26 (online Technical Appendix) was performed as described by Frothingham and Meeker-O'Connell (*12*) on a selection of 20 isolates (Table 2). The isolates originated from 10 properties (6 goat herds and 4 cattle farms), each with 2 different spoligotypes detected at a time. At 5 farms, the loss of spacers 25–27, 29, and 34–38, which can be explained by a single deletion event, had caused a change of the spoligotype pattern. This loss changed SB0157 to SB1081 and SB1084 to SB1889, while the variable number tandem repeat profiles within the same farm remained identical.

The routine application of molecular diagnosis and typing techniques in clinical laboratories has enabled its real role as a pathogen for several species to be recognized. In Spain, *M. caprae* represents 7.4% of all *M. tuberculosis* complex isolates from domestic and wild animals. Seventy-five of the 197 outbreaks (38.1%) involved goats (Table 1). This species showed the highest diversity among *M. caprae* with 12 patterns identified, 6 of them exclusive to caprine herds. The association of *M. caprae* with goats in Spain may be due to 2 reasons. First, the microorganism seems to be highly pathogenic for the goats in Spain, based on the

Table 1. Spoligotyping results of 791 *Mycobacterium caprae* isolates and their distribution within different animal species, Spain, 1992–2009*

		No. animals (no. outbreaks)						
Ref	Spoligotyping pattern†	Goats	Sheep	Cattle	Pigs	Wild boar	Red deer	Fox
SB0157	□■□□□□□□□□□□□□□■■■■■□□■■■■■□■□□□□■■■■■□□□□□	204 (44)	1	133 (67)	1	4 (4)	1	
SB0415	□■□□□□□□□□□□□□□■■□■■■■■■■■□■□□□□■■■■■□□□□□	37 (4)	1	8 (4)		4 (3)		
SB0416	□■□□□□□□□□□□□□□■■■■■■■■■■■■□■□□□□■■■■■□□□□□	80 (18)		58 (21)		5 (3)		1
SB0866	□■□□□□□□□□□□□□□■■■■■■■■□□□□□□□□□□□□□□□□□□	1		1	1			
SB0973	□□□□□□□□□□□□□□□■■■■■■□□■■■■□■□□□□□■□■■□□□□□	1						
SB1077	□■□□□□□□□□□□□□□■■■■■■■■■□□□■□□□□■■■■■□□□□□			17 (4)		1		
SB1078	□■□□□□□□□□□□□□□■■■■□□□■■■■□■□□□□■■■■■□□□□□	1		1				
SB1079	□□□□□□□□□□□□□□□■■■■□□□■■■■□■□□□□□■□■■□□□□□	1						
SB1080	□■□□□□□□□□□□□□□□■■■■■■■■■■□□■□□□□■■■■■□□□□□	2 (1)						
SB1081	□■□□□□□□□□□□□□□■■■■■■□□■□□□□□□□□□□□□□□□□□	1		8 (4)				
SB1084	□■□□□□□□□□□□□□□■■□■■■■□■■■■□■□□□□■■■■■□□□□□	211 (1)						
SB1889	□■□□□□□□□□□□□□□■■□■■■■□□□□□□□□□□□□□□□□□□	2 (1)						
SB1872	□□□□□□□□□□□□□□□□□■■■□□■■■■□■□□□□■■■■■□□□□□	1						
SB0418	□■□□□□□□□□□□□□□□■■■■■■■■■■■□■■■■■■■■■■□□□□□			2 (2)				
SB1690	□■□□□□□□□□□□□□□□■■■■■■■□■■■■□■■■■■■■■■■□□□□□			1				
Total		542 (75)	2 (2)	229 (105)	2 (2)	14 (11)	1	1

*Ref, reference. Numbering according to www.Mbovis.org.
†■, presence of spacer ; □, absence of spacer.

DISPATCHES

Table 2. Variable number tandem repeat analysis of isolates from 10 farms that presented mixed *Mycobacterium caprae* infection (different spoligotype patterns), Spain, 1992–2009*

Farm	Animal	Spoligotype	No. alleles at locus							
			ETR-A	ETR-B	ETR-D	QUB3232	QUB 11a	QUB 11b	MIRU 26	MIRU 31
1	Goat	SB0416	4	4	4	8	7	2	5	2
		SB0866	5	3	3	8	7	4	2	4
2	Goat	SB0416	4	3	4	8	7	2	4	2
		SB0157	4	3	4	8	7	2	4	2
3	Goat	SB0416	4	5	5	7	6	4	5	5
		SB0415	5	1	3	8	7	3	5	5
4	Cattle	SB0157	3	3	4	8	7	2	5	2
		SB1081	3	3	4	8	7	2	5	2
5	Cattle	SB0157	4	3	4	3	7	2	5	2
		SB1081	4	3	4	3	7	2	5	2
6	Goat	SB0157	4	3	4	8	7	2	5	2
		SB1078	4	3	4	8	7	2	5	2
7	Goat	SB1084	5	1	3	9	5†	3	5	4
		SB1889	5	1	3	9	5†	3	5	4
8	Cattle	SB0157	4	3	4	8	7	2	5	2
		SB1081	4	3	4	8	7	2	5	2
9	Cattle	SB0416	5	3	3	8	6	4	2	3
		SB0157	4	3	4	8	7	2	5	2
10	Goat	SB0973	4	3	–	–	–	–	–	–
		SB0157	4	3	4	9	–	2	5	–

*–, no amplification.
†Gel band of ≈1,800 bp. Sequencing showed that insertion sequence IS*6110* is inserted within the third repetition of QUB11a.

disseminated tuberculous lesions that it produces and its fast transmission within a herd. Second, caprine herds have not been included in the national eradication campaign (except when coexisting with cattle or as part of some regional programs). Therefore, *M. caprae* infection can spread easily through animal movements, such as purchase for replacement or genetic improvement.

The emergence of this pathogen in cattle has been observed. Cattle were involved in 106 outbreaks (53.3%) during the study period. Since 2004, cattle from 2,218 herds identified in the eradication program have been inspected by bacteriology. The number of cattle properties infected with *M. caprae* represented 0.85%–6.67% of the total number of herds diagnosed with bovine tuberculosis. Temporal trend of *M. caprae* isolates cultured over time was assessed by using the software WINPEPI 9.4 (*13*). The proportion of *M. caprae* isolated from bovine samples has increased consistently during 2004–2009, showing a significant positive trend (p = 0.009, by Mantel trend test) (Figure 2). We observed more *M. caprae* infections in cattle in regions with a high goat density. However, an analysis of the type of farm production shows that 86.7% of *M. caprae*–infected cattle have been raised in farms without any contact with small ruminants. This fact indicates recirculation of the pathogen within and between cattle herds. In countries that are virtually free of animal tuberculosis such as Germany, Austria, and the Czech Republic, a large number of cases in cattle and red deer are caused by *M. caprae*.

Identification of isolates from human patients has shown *M. caprae* as a human pathogen (*3*,*6*,*14*). A recent study suggests that *M. caprae* causes 0.3% of the cases of human tuberculosis in Spain, with SB0157 also being the most dominant spoligotype (*14*). The role of the pathogen as a public health risk is highlighted by lesions that can

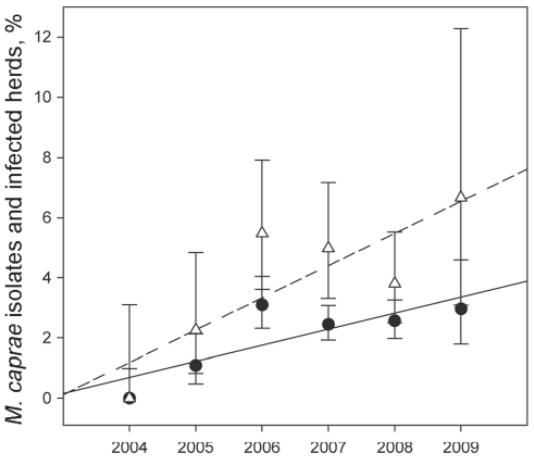

Figure 2. Proportion and regression lines of *Mycobacterium caprae* isolates (black dots, continuous line) and *M. caprae*–infected herds (white triangles, dashed lines) of the total number of *M. tuberculosis* complex isolates and *M. tuberculosis* complex–infected herds identified in cattle during 2004–2009. Error bars indicate 95% confidence intervals.

also be found in the mammary glands of infected goats; thus, consumption of unpasteurized dairy products remains a concern (*15*).

Conclusions

Compelling evidence indicates that *M. caprae* poses a serious health risk not only for goats, but also for other domestic and wild animal species and humans. Our results indicate that *M. caprae* infection is widespread in Spain and that the epidemiology is driven by caprine infections. Considering the role of *M. caprae* in animal tuberculosis, relevant legislation should be considered to address the infection as was done for *M. bovis*.

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Mycobacterium caprae Infection in Livestock and Wildlife, Spain

Technical Appendix

Specific Characteristics

Mycobacterium caprae (*1*), formerly known as *M. tuberculosis* subsp. *caprae* (*2*), and *M. bovis* subsp. *caprae* (*3*) forms a genetically distinct cluster within the *M. tuberculosis* complex. The main features differentiating these isolates from the other members are a special combination of polymorphisms at pyrazinamidase (*pncA*), catalase (*katG*), and subunits A and B of the gyrase (*gyrA* and *gyrβ*) genes (*4*,*5*); the pattern of regions of difference (presence of RD4 and absence of RD5 to 10) (*6*–*8*); and specific patterns obtained by direct variable repeat spacer oligonucleotide typing technique (spoligotyping); and restriction fragment length polymorphism associated with IS*6110*, polymorphic GC-rich sequences, and direct repeat elements (*9*,*10*).

Bacteriology

Tissue samples consisted usually of retropharyngeal, mediastinal, bronchial, and mesenteric lymph nodes, lung and liver. All samples were maintained at –20°C until culture. Samples from each animal were pooled, homogenized with sterile distilled water, decontaminated with 0.35% hexadecylpyridinium chloride for 30 min (*11*), centrifuged at 1,068 × *g* for 30 min, and cultured on Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media (bioMérieux España and Biomedics, Madrid, Spain) at 37°C for 3 mo. The isolates were identified as members of the *M. tuberculosis* complex by PCR amplification of *Mycobacterium* genus–specific 16S rRNA fragment (*12*) and MPB70 sequences (*13*) (primers used in the study are listed in the Table). All PCRs were performed on heat-killed cell suspensions.

Spoligotyping and Data Analysis

The spacer oligonucleotide typing (spoligotyping) method was performed as described by Kamerbeek et al. (*14*). The biotin-labelled amplified product was detected by hybridization onto a spoligotyping membrane (Isogen Bioscience BV, Maarssen, the Netherlands). Hybridized

product was detected with the streptavidin-peroxidase conjugate (Boehringer, Mannheim, Germany) and the electrochemical luminescence system (Amersham, Little Chalfont, UK) by exposing the radiograph film to the membrane. Purified sterile water and a clinical isolate of *M. tuberculosis* and *M. bovis* were included as controls in every batch of tests.

The spoligotyping results were enlisted in a Microsoft Office Access (Microsoft, Redmond, WA, USA) database along with the epidemiologic data (isolation date, animal species and geographical origin). The index of discrimination (D) described by Hunter and Gaston (*15*) was calculated to determine the discriminatory power of the spoligotyping at a national level. We used the website of the University of the Basque Country (www.insilico.ehu.es), filling in the number of unrelated strains for each spoligotype. For this purpose we only counted 1 spoligotype when isolates of the same herd or a precise geographical area shared identical patterns.

Detection of RD4 and Gene Polymorphisms

We used the 3-primer PCR described by Mostowy et al. (*16*). Purified sterile water and a clinical isolate of *M. bovis* were included as controls. The presence (545-bp gel band) or absence (210-bp gel band) of RD4 was detected by agarose gel electrophoresis.

The complete *pncA* gene (*17*) and a part of the *gyrB* (*18*) containing the expected polymorphism for *M. caprae* were amplified. The products were purified with the Qiaquick PCR Purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced with the DyeDeoxy (dRhodamine) Terminator Cycle Sequencing kit in an automatic ABI Prism 373 DNA sequencer (Applied Biosystems, C.I.B. Sequencing Facilities, Madrid, Spain). The sequences generated were aligned with published mycobacterial sequences from the GenBank database (www.ncbi.nlm.nih.gov/GenBank, accession nos. U59967 [*17*] and L27512 [*18*]). Sequencing of the *pncA* demonstrated a C at nucleotide 169, a common characteristic for *M. tuberculosis*, *M. africanum, M. microti,* and *M. caprae* that results in the functional wild-type *pncA* (*17*). The *gyrβ* gene sequence polymorphisms analysis detected, as well the characteristic profile for *M. caprae* that consists of a G at nucleotide 1311 and a C at position 1410, are common to caprine strains and the other members of the complex, except *M. bovis* (*5*).

Variable Number Tandem Repeat Analysis

The PCR for each locus was carried out by using the HotStar Taq DNA polymerase kit (QIAGEN) in a Bio-Rad (Hercules, CA, USA) MyCycler Thermal Cycler. Genomic DNA from

M. bovis BCG Danish was used as a positive control, reaction mixtures lacking mycobacterial DNA were used as a negative control. The number of tandem repeats (alleles) was determined by estimating the amplicon size of the PCR product by electrophoresis on 2.5% agarose gel at 45V for 3 h with a 100-bp ladder (Biotools, B&M Labs, Madrid, Spain).

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Table. List of primers used in a study of *Mycobacterium caprae* infection in livestock and wildlife, Spain*

Target†	Primer	Sequence, 5′ → 3′	Product, bp	Reference
16S rRNA	MYCGEN-F MYCGEN-R	AGAGTTTGATCCTGGCTCAG TGCACACAGGCCACAAGGGA	1,030	*(12)*
MPB70	TB1-F TB1-R	GAACAATCCGGAGTTGACAA AGCACGCTGTCAATCATGTA	372	*(13)*
DR spoligotyping	DR-a DR-b	GGTTTTGGGTCTGACGAC CCGAGAGGGGACGGAAAC	ladder	*(14)*
RD4	RD4-L RD4-R RD4-wtR	GAACGCGACGACCTCATATTCC CTAAGATATCCGGTACGCCCGC CTGTGGCTATGGGGCTCTAC	545/210 (presence/ absence)	*(6,16)*
pncA	pncATB-1 pncATB-2	ATGCGGGCGTTGATCATCGT TCAGGAGCTGCAAACCAACTC	574	*(4,17)*
gyrB	MTUBf MTUBr	TCGGACGCGTATGCGATATC ACATACAGTTCGGACTTGCG	1,020	*(5,18)*
VNTR2165 (ETR-A)	ETRA-F ETRA-R	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT	†	*(19)*
VNTR2461 (ETR-B)	ETRB-F ETRB-R	GCGAACACCAGGACAGCATCATG GGCATGCCGGTGATCGAGTGG	†	*(20)*
VNTR580 (ETR-D, MIRU 4)	ETRD-F ETRD-R	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC	†	*(19,21)*
VNTR3192 (ETR E, MIRU 31)	MIRU31-F MIRU31-R	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT	†	*(22)*
VNTR2996 (MIRU 26)	MIRU26-F MIRU26-R	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG	†	*(21)*
VNTR2163a (QUB11a)	QUB11a-F QUB11a-R	CCCATCCCGCTTAGCACATTCGTA TTCAGGGGGGATCCGGGA	†	*(23,24)*
VNTR2163b (QUB11b)	QUB11b-F QUB11b-R	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT	†	*(23,24)*
VNTR3232 (QUB3232)	3232-F 3232-R	CGGCGATGGTGCCGCCATG CTTGGTGAAGGCCCCGATG	†	*(21)*

*VNTR, variable number tandem repeat; MIRU, mycobacterial interspersed repetitive unit.
†According to respective allele calling tables.

I.3. Dendrograms of *M. bovis* and *M. caprae* spoligotypes from Spain

The following dendrograms represent the group of spoligotypes of *M. bovis* in Spain with spacer 21 present in their spoligotype pattern (Figure 17), the largest group of spoligotypes of *M. bovis* in Spain characterised by the absence of spacer 21 (Figure 18) and the spoligotype patterns of *M. caprae* isolates (Figure 19). The dendrograms were constructed using the application on the MIRU-VNTR plus website (http://www.miru-vntrplus.org) with the unweighted pair group method with arithmetic mean (UPGMA) with default settings (distance measure: categorical; weighting: 1).

The use of phylogenetic trees with spoligotyping profiles is not ideal since the DR locus evolves unidirectionally, and moreover, frequently shows homoplasies (absence or presence of certain spacers in different lineages). Nevertheless, a visualization of spoligotypes on trees can result useful if it is based on known spoligotype signatures and the knowledge of lineage assignation. Therefore, we included information on the membership of distinct spoligotypes to the clonal complexes European 1 (Eu1) and European 2 (Eu2) when available. Phylogenetic trees might reveal possible relationships between the different spoligotype patterns, clustering strains with similar spacer deletions and thus facilitating the analysis of a high number of different patterns.

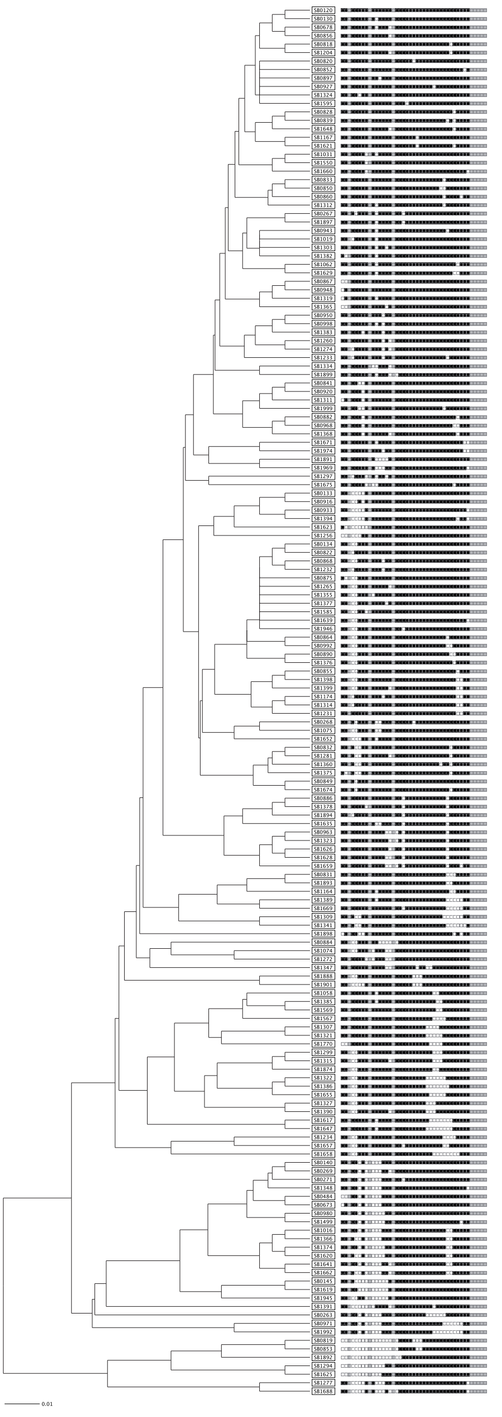


Fig. 17

Figure 17. Dendrogram showing 164 Mycobacterium bovis spoligotypes, with spacer 21 present in their pattern, isolated in Spain. The tree was built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as available on the MIRU-VNTR plus website (www.miru-vntrplus.org). The black/white boxes indicate presence/absence of spacers, the grey boxes mark spacers 3, 9, 16 and 39-43 which are absent from all M. bovis strains. ○absence of Region of Difference (RD) European 1 (Eu1) was confirmed in selected strains. Absence of both, spacer 11 and RDEu1, marks the membership of the Eu1 clonal complex of M. bovis.

Figure 18. Dendrogram showing 259 spacer 21-deleted Mycobacterium bovis spoligotypes isolated in Spain. The tree was built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as available on the MIRU-VNTR plus website (www.miru-vntrplus.org). The black/white boxes indicate presence/absence of spacers, the grey boxes mark the spacers 3, 9, 16 and 39-43 which are absent from all M. bovis strains and spacer 21 which is absent from all the selected spoligotype patterns. ●/○, presence/absence of RDEu1 or the SNP in guaA in selected strains. The absence of spacer 11 and RDEu1 characterises the Eu1 clonal complex of M. bovis. The absence of spacer 21 and the presence of the SNP in guaA mark the membership of the Eu2 clonal complex of M. bovis.

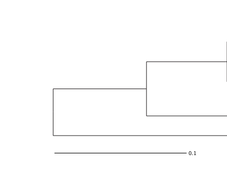


Fig. 19

Figure 19. Dendrogram showing 15 Mycobacterium caprae spoligotypes found in Spain. The tree was built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as available on the MIRU-VNTR plus website (www.miru-vntrplus.org). The black/white boxes indicate presence/absence of spacers, the grey boxes mark spacers 1, 3-16, 28 and 39-43 which are absent from all M. caprae strains.

Fig. 18 ▶

I.4 Contributions to conferences and meetings of European projects

Rodríguez S., Aranaz A., Romero B., de Juan L., Bezos J., Álvarez J., Castellanos E., Moya N., Lozano F.J., Mateos A., Domínguez L. Spoligotyping diversity of *Mycobacterium bovis* in Spain. Oral presentation. Workshop "VNTR/MIRU and DVR-spoligotyping for *Mycobacterium bovis* typing" of European project SSPE-CT-2004-501903. Toledo (Spain), 19-21 October 2006.

Rodríguez S., Castellanos E., Bezos J., Aranaz A., de Juan L., Lozano F., Mateos A. and Domínguez L. The usefulness of DVR-spoligotyping in characterizing Spanish isolates of the zoonotic agents *Mycobacterium bovis* and *Mycobacterium caprae*. Poster. 3rd Med-Vet-Net Annual Scientific Meeting. Lucca (Italy), 27-30 June 2007.

Rodríguez S., Bezos J., de Juan L., Romero B., Álvarez J., Castellanos E., González S., Sáez J. L., Mateos A., Domínguez L. and Aranaz A. Molecular epidemiology underlines the importance of *Mycobacterium caprae* in livestock and wildlife. Oral presentation. 31st Annual Congress of the European Society of Mycobacteriology. Bled (Slovenia), 4-7 June 2010.

Spoligotyping diversity of *Mycobacterium bovis* in Spain

Rodríguez S., Aranaz A., Romero B., de Juan L., Bezos J., Álvarez J., Castellanos E., Moya N., Lozano F.J., Mateos M. and Domínguez L.
Laboratory VISAVET, Departamento Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain.

Spacer oligonucleotide typing (spoligotyping) is a usefool tool for molecular typing of *Mycobacterium bovis*. In this study we analysed 4210 isolates of *M. bovis* isolated in Spain between 1992 and 2006 by DVR-spoligotyping. The strains have been isolated from a wide range of domestic animals, wildlife, zoo animals and pets. A large part of the isolates was cultured in the "Laboratorio de Vigilancia Sanitaria Veterinaria" of the "Universidad Complutense" of Madrid and about 38% of the isolates were cultured and submitted by official laboratories of the communities as part of the bovine tuberculosis control program in Spain.

DVR-spoligotyping was performed as described by Kamerbeek *et al.* (1997). The results clustered the isolates into 210 spoligotypes. The frequency for each spoligotype was calculated, so that spoligotype patterns could be divided into the thirteen most frequent ones, 115 spoligotypes with an average prevalence and 82 so-called orphans. The most frequent patterns make up 6,2% of the spoligotypes. SB0121 has an outstanding prevalence of 25,6% and was found allover Spain. By comparison of the origin of the isolates we found out that some spoligotypes only appear in determined regions. So do SB0339, the predominating strain in El Pardo (Madrid), and SB0135 which was exclusively found in Cantabria.

The large diversity of spoligotypes in Spain permits us to use spoligotyping at a large scale for epidemiological studies, yet the discriminatory power is calculated 0.86. Due to spoligotyping we could establish a relationship between *M. bovis* infecting wildlife and domestic animals in the same geographic area, and, furthermore, analyse relationships between animals of the same or neighbouring farm.

The usefulness of DVR-spoligotyping in characterising Spanish isolates of the zoonotic agents *Mycobacterium bovis* and *Mycobacterium caprae*.

Rodríguez S., Castellanos E., Bezos J., Aranaz A., de Juan L., Lozano F., Mateos A. and Domínguez L.

Laboratory VISAVET, Departamento Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain.

Since the last century, the importance of *Mycobacterium bovis* and *M. caprae* for human tuberculosis has been recognised, leading to implementation of hygienic measures. These pathogens are the main causative agents of tuberculosis in cattle, goats and many other animal species. In recent years several cases of human tuberculosis caused by *M. bovis* and *M. caprae* have been described.

Presently, spacer oligonucleotide typing (spoligotyping) is the most commonly used molecular typing technique for *M. bovis* and *M. caprae* strains as it is cost-effective and easy to reproduce. Between 1992 and 2006 4732 *M. bovis* isolates and 488 *M. caprae* animal isolates from different geographic areas in Spain by DVR-spoligotyping. The technique has been performed as described by Kamerbeek *et al.* 1997 (*J. Clin. Microbiol.* 35:907-14).

The results clustered the isolates into 265 spoligotypes. The frequency for each spoligotype was calculated, so that spoligotype patterns could be divided into: a) the most frequent; b) those of average prevalence; and c) so-called orphans (spoligotypes isolated from only one strain). The most frequent pattern - SB0121 - had an outstanding prevalence and was found all over Spain. By comparing the isolates' origin it was found that some spoligotypes only appear in determined regions.

As the discriminatory power of the applied method is 0.86 it is concluded that the wide diversity of spoligotypes in Spain permits the use of large-scale spoligotyping for epidemiological studies. Furthermore, spoligotyping enabled the establishment of a relationship between *M. bovis* and *M. caprae* infecting wildlife and domestic animals in the same geographic area and the analysis of relationships between animals of the same or neighbouring farm.

The usefulness of DVR-spoligotyping for characterization of Spanish isolates of the zoonotic agents *Mycobacterium bovis* and *Mycobacterium caprae*

S. Rodríguez, E. Castellanos, J. Bezos, A. Aranaz, L. de Juan, F. Lozano, A. Mateos, L. Domínguez

Dpto. Sanidad Animal, Laboratorio VISAVET. Facultad de Veterinaria, Universidad Complutense Madrid

Since the last century, the importance of *Mycobacterium bovis* and *M. caprae* (1) for human tuberculosis has been recognized. These pathogens are the main causative agents of tuberculosis in cattle, goats and many other animal species. In recent years, several cases of human tuberculosis caused by *M. bovis* and *M. caprae* have been described. Direct variable repeat spacer oligonucleotide typing (DVR-spoligotyping), is the most commonly used molecular typing technique for *M. bovis* and *M. caprae* strains as it combines good reproducibility with cost-effectiveness. The objective of this study was to determine the prevalences and the geographic distribution of the Spanish strains and its discriminatory power.

Material and methods

This study comprises 4120 *M. bovis* and 501 *M. caprae* isolates from Spain, collected between 1992 and December 2006. The isolates were obtained from a wide range of animal species, as shown in Fig. 1, from nearly all Spanish regions. The samples were cultured onto Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media (bioMérieux España and Biomedics, Madrid, Spain) at 37ºC. The DNA was prepared from colonies by boiling for 10 min at 100ºC. The isolates were confirmed as members of the *M. tuberculosis* complex by acid-alcohol-fast staining and PCR amplification of *Mycobacterium* genus-specific 16S rRNA fragment and MPB70 sequences (2). DVR-spoligotyping was performed following the protocol described by Kamerbeek et al. (3). Authoritative names for spoligotype patterns were obtained from www.mbovis.org. Our internal nomenclature consists of the prefix spb or spc for classical *M. bovis* or *M. caprae* strains, and a number following a correlative order.

Fig. 1: Number of *M. bovis* / *M. caprae* isolates regarding animal species (others: *M. bovis* isolates from cats (n=3), chamois(n=2), dog (n=1), moufon (n=1)).

Results

- The results clustered the isolates into 210 *M. bovis* and 11 *M. caprae* spoligotypes.
- The frequency for each spoligotype was calculated (Fig. 2 and 3).
- The most frequent *M. bovis* pattern, SB0121, has an outstanding prevalence of 25,6% and was found all over Spain and in nearly all animal species.
- The most frequent *M. caprae* pattern, SB1084, has a prevalence of 49,5% and was only isolated from goats in the region of Madrid.
- By comparison of the origin of the isolates we found out that while some spoligotypes only appear in determined regions other ones can be found all over Spain (Fig. 4 and 5).
- 83 spoligotypes are so-called orphans, which were isolated from only one strain.

Fig. 2. The most frequent *M. bovis* spoligotypes are: SB0121, SB0134, SB0339, SB0265, SB0295, SB0120, SB1232, SB0130, SB0119, SB0135, SPB038, SB0152, SPB-169.

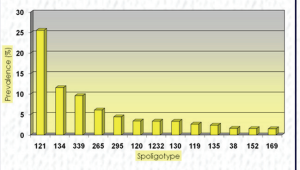


Fig. 3. The *M. caprae* spoligotypes are: SB1084, SB0157, SB0416, SB0415, SPC-016, SB1077, SB1078, SB1081, SB0866, SPC-020, SB1083.

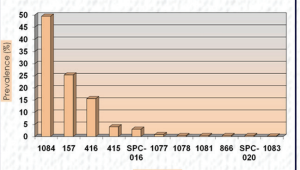


Fig. 4. Geographic distribution of the most frequent spoligotypes in Spain.

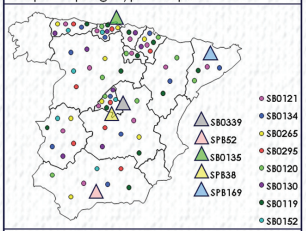
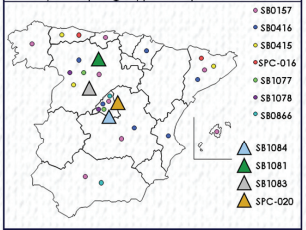


Fig. 5. Geographic distribution of the *M. caprae* spoligotypes in Spain.



- We calculated the discriminatory power by using the equation described by Hunter and Gaston (4):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

- The discriminatory index for *M. bovis* strains was 0,86 and for *M. caprae* strains 0,62.

Conclusions

- Due to the high discriminatory index we conclude that the large diversity of *M. bovis* spoligotypes in Spain permits the use of spoligotyping at a large scale for epidemiological studies.
- Furthermore, spoligotyping has allowed us to establish a relationship between *M. bovis* and *M. caprae* infecting wildlife and domestic animals in the same geographic area, and to analyze relationships between animals of the same or neighbouring farm.
- For *M. caprae* isolates it might be useful to apply other molecular typing techniques in order to achieve a higher discrimination.
- It is very important to centralize the information on Spanish *M. bovis* and *M. caprae* isolates. Therefore we are establishing a national database within a project with the Spanish Ministry for Agriculture, Food and Fisheries .

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4. Hunter and Gaston, 1988. *J.Clin.Microbiol.*, 26, S. 2465-2466.

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Molecular epidemiology underlines the importance of *Mycobacterium caprae* in livestock and wildlife

Rodríguez S. [1,2], Bezos J. [1,2], de Juan L.[1,2], Romero B. [1,2], Álvarez J.[1], Castellanos E. [1,2], González S.[1], Sáez J. L. [3], Mateos A. [1,2], Domínguez L. [1,2] and Aranaz A. [1,2]

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[3] Spanish Ministry of the Environment, and Rural and Marine Affairs.

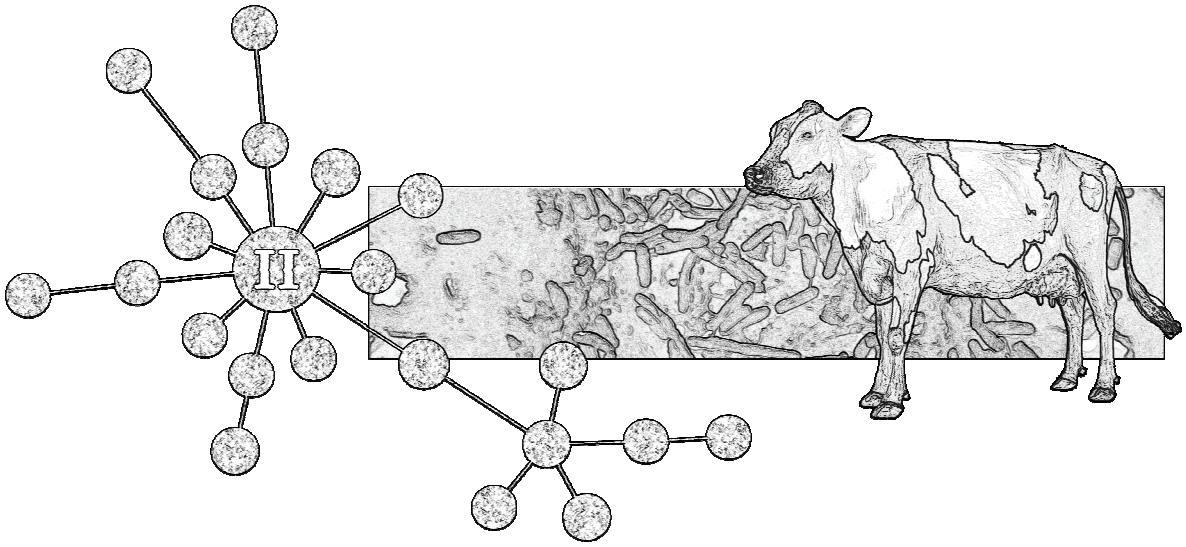
Mycobacterium (*M.*) *caprae* is a member of the *M. tuberculosis* complex which was first described as the main etiological agent of caprine tuberculosis in Spain. However, this pathogen can infect other animal species and human beings.

In the present study we have characterised *M. caprae* isolates from 791 animals by DVR-spoligotyping. The diversity of spoligotypes based on the number of patterns (n=15) and discrimination index (D=0.85) is lower compared to *M. bovis*. Additionally, we used Variable Number Tandem Repeat (VNTR) typing when more than one spoligotype was observed in the same herd. *M. caprae* infection was widespread in Spain and although the majority of the strains (n=536) were identified in goats, we also observed *M. caprae* in other domestic and wil animal species [cows (n=235), sheep (n=2), pigs (n=2), wild boars (n=14), red deer (n=1) and fox (n=1)]. Proportion of *M. caprae* isolates from bovine samples increased consistently in the period 2004-2009, highlighting an emergence of this pathogen in cattle.

Unlike results found in reports from other European countries, we observed that the epidemiology in Spain is driven by caprine infections. Considering the damage that *M. caprae* causes in caprine flocks, the possibility of its transmission to other animal species and its zoonotic potential, we suggest that relevant legislation should be adapted to address the infection as it is done with *M. bovis*.

Chapter II

Molecular typing as a tool in tracking outbreaks caused by *Mycobacterium bovis*



Molecular typing as a tool in tracking outbreaks caused by *M. bovis*

Bovine tuberculosis remains a concern on national and international level and its control and surveillance have become reinforced by the development and large-scale application of genotyping techniques such as Direct Variable Repeat (DVR) spoligotyping (Kamerbeek *et al.*, 1997) and mycobacterial interspersed repetive unit-variable number tandem repeat (MIRU-VNTR) typing (Frothingham and Meeker-O'Connell, 1998; Supply *et al.*, 2000). The most common technique applied to *M. bovis* isolates is spoligotyping, but in some countries or determined geographical settings the discrimination achieved with this method is insufficient (Skuce *et al.*, 2005; Hewinson *et al.*, 2006; Smith *et al.*, 2006). Consequently, spoligotyping is complemented with MIRU-VNTR typing that aims at different loci all over the genome in order to increase the discrimination and enable the assessment of relationships between strains and even possible sources of infection.

Whereas the public health sector working with *M. tuberculosis* has achieved a consensus on the combination of loci for MIRU-VNTR typing, the sets of MIRU-VNTR markers used for typing *M. bovis* isolates remain diverse. A combination of six markers (ETR-A, ETR-B, ETR-D, QUB11a, QUB11b and QUB3232) has been proposed by VENoMYC (EU coordination action SSPE-CT-2004-501903; Supply, 2006), but allelic diversities, and hence the discriminatory power, of the markers vary between countries and therefore individual combinations of markers are currently used. In Spain, apart from the six before mentioned markers, another three loci have been used for typing *M. bovis* isolates [ETR-E, MIRU26 (Gortázar *et al.*, 2005); QUB26 (Romero *et al.*, 2008)]. In order to improve our knowledge about the relationships between the Spanish *M. bovis* strains, the degree of diversity in terms of MIRU-VNTR typing and to exploit this technique for the tracking of outbreaks, three different panels of *M. bovis* isolates were assessed using the nine MIRU-VNTR markers mentioned above.

The first study was directed in 115 *M. bovis* isolates with spoligotype SB0121, the most frequent spoligotype in Spain. MIRU-VNTR typing with nine loci resulted in 65 different profiles, yielding an index of discrimination (D) (Hunter and Gaston, 1988; Hunter, 1990) of 0.9856. An expansion of a clonal group of closely related strains could be observed that was maintained when the set of markers was reduced to the four most discriminatory loci (ETR-A, -B, QUB11a and -3232) achieving 51 MIRU-VNTR types and a D of 0.9676. The distinct MIRU-VNTR types do not cluster in specific geographic areas in contrast to observations from the UK (Smith *et al.*, 2006). The high diversity observed in these isolates is congruent with findings in Portugal (Cunha *et al.*, 2011) underlining the close relationship of the strains in the Iberian Peninsula.

The second study aimed at the identification of the genotype of *M. bovis* that caused the first outbreak in alpacas in Spain. The isolates from the three deceased animals showed an identical genotype [spoligotype SB0295, MIRU-VNTR type 6-4-3-4-5-11-2-5-6 (in the following order: ETR-A, -B, -D, -E, MIRU26, QUB11a, -11b, -26 and -3232); QUB11a could not be amplified in one of the isolates]. Subsequently, possibly related *M. bovis* isolates within the area around the two affected alpaca farms were selected and 47 isolates with matching spoligotype, SB0295, or closely related spoligotypes, SB0121 and SB1190, were MIRU-VNTR typed at the nine loci. None of the 11 identified MIRU-VNTR types matched the profile of the causative agent of the alpaca tuberculosis. However, all the genotypes were closely related and clustered in a clonal group and a linked subgroup, except from five singletons that in comparison to the other profiles varied at more than one locus.

The third panel under study was composed of 39 *M. bovis* isolates originating from bullfighting properties containing 34 isolates from bullfighting cattle and five from wildlife which were likewise typed using the nine MIRU-VNTR markers. The genotyping results from this study underline the circulation of the most frequent spoligotypes in Spain in bullfighting cattle and wildlife living on the same premises and interestingly, identified a dominant genotype [spoligotype: SB0295, MIRU-VNTR type: 6-4-3-3-5-10-2-5-7 (order as above)].

In the course of these studies *M. bovis* BCG Danish (CCUG 27863, Culture Collection, University of Göteborg, Sweden) was used as a positive control and was sequenced at each of the nine loci using Applied Biosystems™ ABI Prism 3730 DNA Sequencer (CIB Sequencing Facilities, Madrid, Spain) and a correlating allele calling table (Table 8) was built according to the sequencing results and previous publications (Frothingham and Meeker O'Connell., 1998; Supply *et al.*, 2000; Roring *et al.*, 2002; Skuce *et al.*, 2002).

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Table 8. Allele calling table for the nine variable number tandem repeat (VNTR) loci used for the VNTR typing studies. The results for the positive control *Mycobacterium bovis* BCG Danish (CCUG 27863, Culture Collection, University of Göteborg, Sweden) at each locus is shown in bold.

VNTR LOCUS (alias)	R[a]	P[b]	FL[c].	Size (bp) corresponding to the number of repeats:																
				0'[d]	0[e]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
2165 (ETR-A)	75	23	195	172	195	270	345	420	495	**570**	645	720	795	870	945	1020	1095	1170	1245	1320
2461 (ETR-B)	57	6	121	115	121	178	235	292	349	**406**	463	520	577	634	691	748	805	862	919	976
580 (ETR-D, MIRU4)	77	53	122/175[f]	122	175	252	329	406	483	560	637	714	791	868	945	1022	1099	1176	1253	1330
				0'	-	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'	11'	12'	13'	14'	15'
				122	-	199	**276**	353	430	507	584	661	738	815	892	969	1046	1123	1200	1277
3291 (ETR-E, MIRU31)	53	2	491	489	491	544	597	**650**	703	756	809	862	915	968	1021	1074	1127	1180	1233	1286
2996 (MIRU26)	51	8	285	277	285	336	387	438	489	**540**	591	642	693	744	795	846	897	948	999	1050
2163a (QUB11a)	69	15	167	152	167	236	305	374	443	512	581	650	719	788	**857**	926	995	1064	1133	1202
2163b (QUB11b)	69	9	67	58	67	136	205	**274**	343	412	481	550	619	688	757	826	895	964	1033	1102
4052 (QUB26)	111	24	153	129	153	264	375	486	597	**708**	819	930	1041	1152	1263	1374	1485	1596	1707	1818
3232 (QUB3232)[g]	56	48	181	133	181	237	293	349	405	**461**	517	573	629	685	741	797	853	909	965	1021

[a] Size of repeat (bp)
[b] Size of partial repeat (bp)
[c] Size of the flanking region (bp)
[d] Flanking region without partial repeat
[e] Flanking region with partial repeat.
[f] *M. bovis* BCG and approximately 1% of *M. tuberculosis* isolates lack the partial repeat of 53 bp at VNTR locus 580; in these cases the number of repeats is expressed as prime (').
[g] The size corresponding to the number of repeats is based on amplification of the short fragment of VNTR locus 3232.

II.1 Discrimination of VNTR typing rises with the expansion of a clonal group of *M. bovis*

Discrimination of Variable Number Repeat Typing rises with the expansion of a clonal group of *Mycobacterium bovis*

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Abstract

Mycobacterium bovis populations usually show a more prevalent spoligotype with a wide geographical distribution. This study applies Mycobacterial Interspersed Repetive Unit (MIRU) -Variable Number Tandem Repeat (VNTR) typing to a representative panel of 115 *M. bovis* isolates with the most frequent spoligotype in the Iberian Peninsula, SB0121. VNTR typing targeted ETR-A (VNTR2165), ETR-D (MIRU4, VNTR580), QUB11a (VNTR2163a), QUB11b (VNTR2163b), ETR-B (VNTR2461), ETR-E (MIRU31, VNTR3192), MIRU26 (VNTR2996), QUB26 (VNTR4052) and QUB3232 (VNTR3232). The practical use of the nine markers and different combinations of these was assessed calculating the discriminatory indexes and building minimum spanning trees that show a hypothetical expansion of a clonal group. We found a high diversity among isolates of the panel (D = 0.9856) and conclude that a less stringent interpretation could be applied for disease tracking which means to consider single or double allele variants as possible sources.

Introduction

Bovine tuberculosis caused by *Mycobacterium bovis* (*M. bovis*) remains a concern in Spain, although the herd prevalence, 1.51% in 2010 (Ministry of the Environment, and Rural and Marine Affairs), has been drastically reduced since systematical implementation of the national eradication program in 1990s (Report Spain - 2009; http://www.aesan.mspsi.gob.es/AESAN/docs/docs/control_oficial/planes_nacionales_especificos/Informe_fuentes_2009_ESPANA.pdf). In consideration of the economic and public-health implication of this zoonosis, tracing the source of infections has become a key element within the program, and genotyping has therefore become a powerful tool. The most widely used technique to type *M. bovis* isolates is Direct Variable Repeat (DVR) spoligotyping (Kamerbeek et al., 1997). However, in some countries or distinct geographical settings this cost-effective high-throughput technique does not offer an adequate discrimination (Skuce et al., 2005; Hewinson et al., 2006; Smith et al., 2006) and is thus complemented with Mycobacterial Interspersed Repetive Unit (MIRU) - Variable Number Tandem Repeat (VNTR) typing (Frothingham and Meeker-O'Connell, 1998; Supply et al., 2000).

In recent surveys from Portugal (Duarte et al., 2008) and Spain (Rodriguez et al., 2010) SB0121 was found to be the most frequent spoligotype (26.3% and 27.9%, respectively). These studies also coincided in high levels of discrimination achieved by spoligotyping (D=0.89 and 0.87, respectively) and subsequent studies from Portugal found the discrimination increased with MIRU-VNTR typing (Duarte et al., 2010; Cunha et al., 2011).

In this study we have used nine MIRU-VNTR markers for the typing of a panel

of 115 Spanish *M. bovis* isolates with spoligotype SB0121. The allelic diversity and overall discriminatory power of the technique are evaluated and we discuss how high levels of diversity influence the interpretation of the results in terms of molecular epidemiology.

2. Material and Methods

2.1. Mycobacterial strains

The study was carried out in a panel of 115 *M. bovis* isolates (Supplementary Table) with spoligotype SB0121. These comprised 111 isolates selected randomly from the Spanish strain collection at the Centre VISAVET originating from cattle (*Bos taurus*, n=98), goats (*Capra aegagrus hircus*, n=2), wild boar (*Sus scrofa*, n=7), red deer (*Cervus elaphus*, n=2), fallow deer (*Dama dama*, n=2) and four additional *M. bovis* isolates from less frequently affected species badger (Meles meles, n=2 from a recent report (Balseiro et al., 2011) and domestic pig (*Sus scrofa domestica*, n=2)]. Isolates were obtained from sampling between 1997 and 2010 from all over Spain.

Spoligotyping was perfomed following the protocol described by Kamerbeek et al. (1997) and authoritative names (prefix SB followed by four digits) assigned according to the *Mycobacterium bovis* Spoligotype Database website http://www.Mbovis.org (Smith and Upton, 2011).

2.2. Variable Number Tandem Repeat (VNTR) Typing

VNTR typing was performed following the protocol described by Frothingham and Meeker-O'Connell (Frothingham and Meeker-O'Connell, 1998) targeting nine VNTR loci: ETR-A (VNTR2165), ETR-D (MIRU4, VNTR580), QUB11a

(VNTR2163a), QUB11b (VNTR2163b) (Allix et al., 2006; Supply, 2006), ETR-B (VNTR2461) (Frothingham and Meeker-O'Connell, 1998), ETR-E (MIRU31, VNTR3192), MIRU26 (VNTR2996) (Supply et al., 2001), QUB26 (VNTR4052) (Skuce et al., 2002) and QUB3232 (VNTR3232) (Supply, 2006). Six of these markers (ETR-A, B, D, QUB11a, 11b and 3232) were recommended for typing of *M. bovis* by VENoMYC (EU coordination action SSPE-CT-2004-501903; Supply, 2006) and the other additional three loci have been previously used for typing Spanish strains [ETR-E, MIRU26 (Gortázar et al., 2005); QUB26 (Romero et al., 2008)]. The PCRs were performed using the HotStarTaq DNA polymerase kit (Qiagen) in a Bio-Rad MyCycler Thermal Cycler. Reaction mixtures without mycobacterial DNA were used as a negative control Genomic DNA and *M. bovis* BCG Danish (CCUG 27863, Culture Collection, University of Göteborg, Sweden) was used as a positive control. The positive control had been sequenced at each of the nine loci using Applied Biosystems™ ABI Prism 3730 DNA Sequencer (CIB Sequencing Facilities, Madrid, Spain) and a correlating allele calling table was built according to previous publications (Frothingham and Meeker O'Connell., 1998; Supply et al., 2000; Roring et al., 2002; Skuce et al., 2002). The number of tandem repeats (alleles) was estimated after electrophoresis on 2.5% agarose gels at 45 V during 5 h with a 100 bp ladder (Biotools B&M Labs, Madrid, Spain) according to the allele calling table.

2.3. Data analysis

The index of discrimination (D) (Hunter and Gaston, 1988; Hunter, 1990) was calculated in order to determine the overall discriminatory power of the MIRU-VNTR typing technique as well as the individual allelic diversity of the nine loci. We used the in-silico website of the University of the Basque Country (http://www.insilico.ehu.es),

filling in the number of unrelated isolates with each MIRU-VNTR type or allele, respectively. The MIRU-VNTR plus website (www.miruvntrplus.org) was used for the construction of the minimum spanning trees (Allix-Béguec et al., 2008; Weniger et al., 2010); loci QUB11a and QUB3232 are not included in the standardised datasets of MIRU-VNTR plus and have therefore been arbitrarily assigned to database fields of standard loci.

3. Results

MIRU-VNTR typing using nine markers (ETR-A, -B, -D, -E, MIRU26, QUB11a, 11b, 26 and 3232) divided the 115 *M. bovis* isolates with spoligotype SB0121 into 65 different MIRU-VNTR types (Supplementary Table). We observed the following allelic diversities for the different loci (Table 1): QUB3232 (D=0.83), ETR-A (D=0.65), ETR-B (0.53), QUB11a (D=0.45), QUB26 (0.38), MIRU26 (0.35), ETR-D (0.20), MIRU31 (0.17) and QUB11b (0.08). The overall discriminatory power of the MIRU-VNTR typing technique using these nine loci was D=0.9856. The three biggest clusters were MIRU-VNTR (MV) type 1A (9 isolates) followed by MV type 3 (6 isolates), MV type 2A (5 isolates), MV types 5A and 7A (four isolates each), and MV types 4A and 6A (3 isolates each); ten MV types clustered two isolates, and 48 types were unique (Supplementary Table). Using only the four most discriminatory loci (ETR-A, ETR-B, QUB11a and QUB3232) the isolates were clustered in 51 MV types achieving a discrimination of D=0.9676. In this case, the biggest cluster included MV type 1 (16 isolates), MV type 2 (8 isolates), MV types 3 and 4 (6 isolates each) and MV types 5, 6 and 7 (five isolates each), MV type 8 (3 isolates), ten MV types clustered two isolates and 33 types were unique. The combination of seven loci, excluding QUB11a

and QUB3232, yielded 37 MV types and the discrimination dropped to D=0.9404.

Eight isolates (for the four loci approach) and 13 isolates (for the nine loci approach) were excluded from the analysis due to repeated failure of amplification or undetermined alleles (double bands) at one or more loci; the loci which most often presented problems were QUB11a and MIRU31 (Table 1).

The construction of minimum spanning trees for the three different combinations of loci [nine loci and four loci (Figure 1), seven loci not shown] revealed that the MIRU-VNTR types are closely related resulting in expansion of a clonal group. Despite the high diversity, most of the genotypes (n=57, 87.7%) belonged to the same clonal complex considering the maximum difference within a clonal complex variation at two loci (double locus variant = DLV). Using a less stringent criterion, allowing triple locus variants within a clonal complex, all MV types (except from MV type 40, a singleton with variation at 4 loci) cluster in a clonal group. The analysis of the panel with only four loci considering before mentioned criteria grouped all isolates in a single clonal group.

Among the 98 cattle isolates included in the analysis, 15 pairs (30 isolates) were epidemiologically related (from the same farm). Six pairs of the related isolates had different MV types due to variation at one or more of the loci ETR-A, ETR-B, MIRU26, QUB11a, QUB11b, QUB26 or QUB3232, the latter being involved in all cases. In the case of four pairs of related isolates the MV type variation was due to changes at four to seven loci, but two pairs of isolates showed different MV types as a result of variation only at locus QUB3232. Both combinations (nine loci and four loci) differentiate these pairs of related isolates.

The panel also included a small group of isolates from animal species other than cattle which have been previously described as hosts. These isolates clustered either in

157 the largest groups of the MV types or were related to MV types found in cattle. Even
158 with the nine loci approach isolates form cattle and other species seem identical (goat
159 and cattle isolates in MV type 4B, wild boar and cattle in MV type 3, red deer and
160 fallow deer in MV type 9) or closely related by microevolution in a single locus (wild
161 boar MV type 1E, 6B, 18B, and fallow deer MV type 34).
162
163 **4. Discussion**
164
165 The *M. bovis* population in the Iberian Peninsula is dominated by the European 2
166 clonal complex which makes up about 70% of the isolates and is characterised by the
167 loss of spacer 21 and a single nucleotide polymorphism in gene *guaA* (Rodriguez-
168 Campos et al., 2011b). Spoligotype SB0121 is the most prevalent representative of this
169 group and its putative recent common ancestor. This type is also present at high
170 frequency in Portugal (Duarte et al., 2008) but less prevalent in France (Haddad et al.,
171 2001) and Italy (Boniotti et al., 2009). The present study used MIRU-VNTR analysis to
172 determine the degree of diversity within this spoligotype and to assess the reliability of
173 spoligotyping for epidemiological purposes when this highly frequent pattern is found.
174 A common feature of *M. bovis* populations is that each geographical area presents a
175 clearly more frequent spoligotype which can be subtyped by MIRU-VNTR typing to a
176 variable extent. Consistent with a previous report from Portugal (Duarte et al., 2010;
177 Cunha et al., 2011) and Italy (Boniotti et al., 2009) the MIRU-VNTR type diversity of
178 the most prevalent spoligotypes (SB0121 and SB0120, respectively) is high compared
179 to that of the European 1 clonal complex, which is at virtual fixation in the United
180 Kingdom and the Republic of Ireland (Smith et al., 2011). This diversity might result
181 from a higher mutation rate, or it may be due to the earlier introduction of the pathogen

182 in mainland Europe which gave more opportunity for evolution. With such high
183 diversity of genotypes, almost no clustering of distinct MIRU-VNTR types in specific
184 geographic areas can be found, in contrast to a report from the UK (Smith et al., 2006).
185 The underlying reasons for this apparent higher mutation rate in the Iberian *M.*
186 *bovis* population remain unknown. In order to screen for large polymorphisms that
187 could hint at an explanation of the expansion of certain genotypes we analysed three
188 isolates with spoligotype SB0121 with different MV types by DNA microarray (Garcia-
189 Pelayo et al., 2004). These isolates included a cattle isolate with MV type 1A, the
190 largest cluster found in the study, a cattle isolate with MV type 2D, a unique type, and a
191 wild boar isolate with MV type 44, also a unique type, but no such polymorphisms
192 common to the clonal group were identified (unpublished data).
193 In the animal health sector it is especially important to apply cost-effective
194 techniques with an optimised level of discrimination. Obviously, the more markers are
195 included in the analysis, the better resolution of genotypes is achieved. However, in
196 practical terms this is not feasible when handling large collections of isolates. Thus, we
197 consider the use of the four loci ETR-A, ETR-B, QUB11a and QUB3232, sufficient for
198 routine application in *M. bovis* isolates from a high diversity setting. These four loci
199 were among the loci proposed by VENoMYC for the MIRU-VNTR typing of *M. bovis*
200 strains (EU coordination action SSPE-CT-2004-501903; Supply, 2006). The analysis of
201 the panel of isolates with the four loci maintains a reasonable discrimination value and
202 does not change the main structure of the minimum spanning tree, as shown in Figure 1.
203 Nevertheless, we recommend the use of the additional five loci (ETR-D, ETR-E,
204 MIRU26, QUB11b and QUB26) for a more specific use, for example molecular tracing
205 of outbreaks. The use of loci QUB11a and QUB3232 is controversial because they have
206 been previously described as hypervariable (Supply et al., 2006), but also have been

found useful in recent studies in determined settings (Hilty et al., 2005), lineages (Velji et al., 2009) and mycobacterial species, for example *M. bovis* (McLernon et al., 2010; Lari et al., 2011). In this study their use did not seem to affect the data analysis negatively.

The putative founder of the cluster (central node) is MV type 2A (fig. 1) though the pattern is not the most frequent one in the panel (difference at QUB11a and QUB3232), as also described in a large study in the UK (Smith et al., 2003). The contribution of each loci to diversity results of a balance between the number of alleles and the distribution of the isolates at each possible allele, as shown in Table 1. Accordingly, the loci with higher D value are main contributors to the expansion of the clonal group. We have observed that differences between strains in this panel were mainly caused by single or double allele variations. In this snapshot of the population of the most frequent *M. bovis* spoligotype in Spain we did not observe clustering of distinct MIRU-VNTR types in specific geographic areas or animal species with any of the loci combinations.

In high diversity settings, like Portugal and Spain, clonal groups of similar MIRU-VNTR types seem to expand leading to an overly high discrimination which might hamper the identification of sources of infection on perfect match basis as previously observed in an attempt to trace an outbreak in an alpaca farm (Rodriguez-Campos et al., 2011a). We conclude that a less stringent interpretation should be applied for disease tracking which means to consider single or double allele variants as possible sources.

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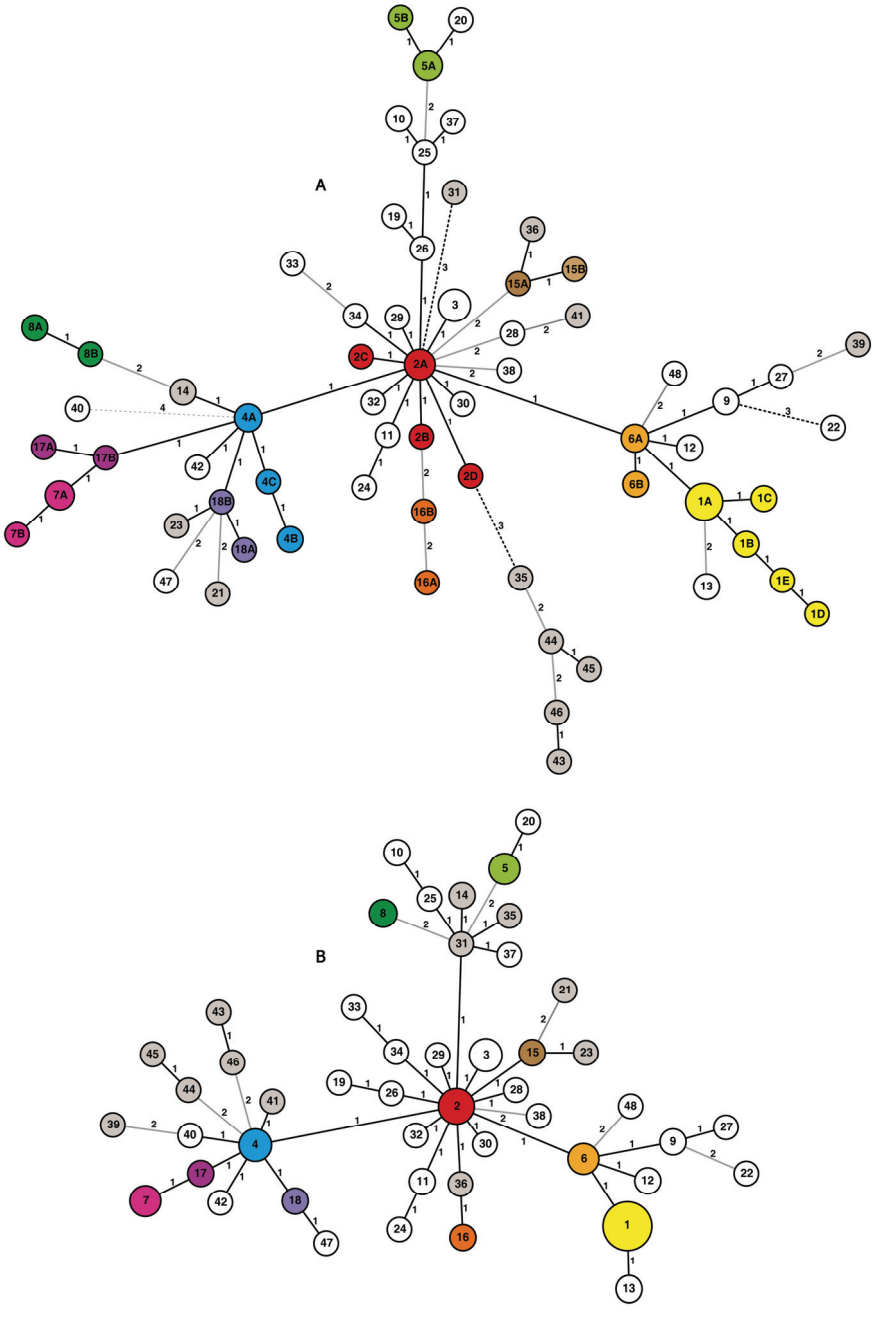
Table 1. Allelic diversity of the individual VNTR loci and the different combinations of these.

MIRU-VNTR locus (alias)	No. of isolates with MIRU-VNTR allele:																	D
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	N[b]	DB[c]	
3232 (QUB3232)				2	6	17	32[a]	30	7	11	5	1	1	2	1	0	0	0.8259
2165 (ETR-A)	1		2	10	49	46[a]	1	1				1				2	2	0.6541
2461 (ETR-B)		9	17	76[a]	11	1										0	1	0.5336
2163a (QUB11a)				1	2	1	9	1	6	83[a]	1	3	1		1	6	0	0.4366
4052 (QUB26)		2	6	11	89[a]	2	1									2	1	0.3786
2996 (MIRU26)		6		6	92[a]	8										3	0	0.3450
580 (ETR-D)		6	104[a]	5												0	0	0.2038
3192 (ETR-E)		4	106[a]													5	0	0.1679
2163b (QUB11b)	3	109[a]	1													2	0	0.0790
9 loci[c]																		0.9856
7 loci[d]																		0.9404
4 loci[e]																		0.9676

[a] No. of isolates at the allele that corresponds to the most frequent MIRU-VNTR type.
[b] Not amplifiable.
[c] Double bands.
[d] Combination of ETR-A, ETR-B, ETR-D, ETR-E, MIRU 26, QUB11a, QUB11b, QUB26 and QUB3232.
[e] Combination of ETR-A, ETR-B, ETR-D, ETR-E, MIRU 26, QUB11b and QUB26.
[f] Combination of ETR-A, ETR-B, QUB11a and QUB3232.

Figure 1. Minimum spanning trees (MST) for the 65 (A) and the 51 (B) MIRU-VNTR types obtained with nine-loci typing and four-loci typing of the 115 *Mycobacterium bovis* isolates, respectively. MSTs were created using the online application MIRU-VNTRplus (Weniger et al., 2010). MIRU-VNTR types that clustered at different central nodes when reducing the set of markers are shown in grey; the coloured nodes show MIRU-VNTR types that were subdivided when the number of markers was increased.



Supplementary Table. Mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing results for the 115 *Mycobacterium bovis* isolates at nine MIRU-VNTR loci: N, not amplifiable, /, double band.

ID[a]	MIRU-VNTR loci[b]									MIRU-VNTR types (9 loci)[c]	MIRU-VNTR types (4 loci)[d]	Animal origin
	ETR-A	ETR-B	ETR-D	ETR-E	MIRU26	QUB11a	QUB11b	QUB26	QUB3232short			
	2165	2461	580	3192	2996	2163a	2163b	4052	3232			
MI05/00611	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI06/00833	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI07/00008	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI08/03982	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI07/10158	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI07/10206	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI07/10142	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI07/10213	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI07/14002	6	4	3	3	5	10	2	5	7	1A	1	cattle
MI06/01507	6	4	3	3	2	10	2	5	7	1B	1	cattle
MI08/00019	6	4	3	3	2	10	2	5	7	1B	1	cattle
MI07/16345	6	4	3	3	5	10	2	3	7	1C	1	cattle
MI08/00936	6	4	3	3	5	10	2	3	7	1C	1	cattle
MI08/06952-1	6	4	3	3	5	10	2	N	7	-	1	goat
MI07/03539	6	4	3	3	2	10	1	2	7	1D	1	cattle
02/0439	6	4	3	3	2	10	2	2	7	1E	1	wild boar
98/0619	5	4	3	3	5	10	2	5	8	2A	2	cattle
98/0620	5	4	3	3	5	10	2	5	8	2A	2	cattle
99/0144	5	4	3	3	5	10	2	5	8	2A	2	cattle
MI06/02508	5	4	3	3	5	10	2	5	8	2A	2	cattle
MI07/01089	5	4	3	3	5	10	2	5	8	2A	2	cattle
MI07/11268	5	4	3	3	4	10	2	5	8	2B	2	cattle
MI07/00704	5	4	3	3	5	10	2	4	8	2C	2	cattle
MI06/00001	5	4	3	3	6	10	2	5	8	2D	2	cattle
03/0549	4	4	3	3	5	10	2	5	8	3	3	cattle
MI07/08592	4	4	3	3	5	10	2	5	8	3	3	cattle
MI07/11655	4	4	3	3	5	10	2	5	8	3	3	cattle

[a] Isolate reference.
[b] MIRU-VNTR loci with corresponding alias.
[c] MIRU-VNTR types obtained with the nine-loci approach.
[d] MIRU-VNTR types obtained with the four-loci approach. Table is arranged according to the number of isolates with each type in descending order.

ID[a]	MIRU-VNTR loci[b]									MIRU-VNTR types (9 loci)[c]	MIRU-VNTR types (4 loci)[d]	Animal origin
	ETR-A	ETR-B	ETR-D	ETR-E	MIRU26	QUB11a	QUB11b	QUB26	QUB3232short			
	2165	2461	580	3192	2996	2163a	2163b	4052	3232			
MI08/06714	4	4	3	3	5	10	2	5	8	3	3	cattle
MI08/06728	4	4	3	3	5	10	2	5	8	3	3	cattle
MI08/11685	4	4	3	3	5	10	2	5	8	3	3	wild boar
MI05/04376	6	4	3	3	5	10	2	5	8	4A	4	cattle
MI06/00214	6	4	3	3	5	10	2	5	8	4A	4	cattle
MI06/07842	6	4	3	3	5	10	2	5	8	4A	4	cattle
MI07/01798-2	6	4	3	3	4	10	2	3	8	4B	4	goat
MI07/04849	6	4	3	3	4	10	2	3	8	4B	4	cattle
MI05/02585	6	4	3	3	5	10	2	3	8	4C	4	cattle
04/1602	5	2	3	3	5	7	2	5	6	5A	5	cattle
MI06/05500	5	2	3	3	5	7	2	5	6	5A	5	cattle
MI07/04842	5	2	3	3	5	7	2	5	6	5A	5	cattle
MI07/08681	5	2	3	3	5	7	2	5	6	5A	5	cattle
MI07/11265	5	2	2	3	5	7	2	5	6	5B	5	cattle
MI05/00804	5	4	3	3	5	10	2	5	7	6A	6	cattle
04/1048	5	4	3	3	5	10	2	5	7	6A	6	cattle
MI07/12798	5	4	3	3	5	10	2	5	7	6A	6	cattle
MI06/05151	5	4	3	3	6	10	2	5	7	6B	6	wild boar
DICM08/00669-3	5	4	3	3	6	10	2	5	7	6B	6	wild boar
MI05/02380	6	5	3	3	5	10	2	5	10	7A	7	cattle
MI06/05819	6	5	3	3	5	10	2	5	10	7A	7	cattle
MI07/10177	6	5	3	3	5	10	2	5	10	7A	7	cattle
MI07/16293	6	5	3	3	5	10	2	5	10	7A	7	cattle
98/0502	6	5	3	3	5	10	2	3	10	7B	7	cattle
03/0098	4	2	2	3	5	10	2	5	6	8A	8	cattle
MI06/00238	4	2	2	3	5	10	2	5	6	8A	8	cattle
04/0265	4	2	3	3	5	10	2	5	6	8B	8	cattle
MI08/12888	5	3	3	3	5	10	2	5	7	9	9	red deer
MI08/04144	5	3	3	3	5	10	2	5	7	9	9	fallow deer
MI05/00319	5	3	3	3	5	9	2	5	6	10	10	cattle

[a] Isolate reference.
[b] MIRU-VNTR loci with corresponding alias.
[c] MIRU-VNTR types obtained with the nine-loci approach.
[d] MIRU-VNTR types obtained with the four-loci approach. Table is arranged according to the number of isolates with each type in descending order.

ID[a]	MIRU-VNTR loci[b]									MIRU-VNTR types (9 loci)[c]	MIRU-VNTR types (4 loci)[d]	Animal origin
	ETR-A	ETR-B	ETR-D	ETR-E	MIRU26	QUB11a	QUB11b	QUB26	QUB3232short			
	2165	2461	580	3192	2996	2163a	2163b	4052	3232			
MI05/00323	5	3	3	3	5	9	2	5	6	10	10	cattle
MI07/16333	5	4	3	3	5	10	2	5	10	11	11	cattle
MI07/01170	5	4	4	N	5	10	2	5	10	-	11	cattle
97/0024	5	4	3	3	5	7	2	5	7	12	12	cattle
MI08/06282	5	4	3	3	5	7	2	5	7	12	12	cattle
MI07/06633	6	3	3	3	5	10	2	4	7	13	13	cattle
MI07/11354	6	3	3	3	5	10	2	4	7	13	13	cattle
MI06/00216	6	4	3	3	5	10	2	5	6	14	14	cattle
MI07/08629	6	4	3	3	5	10	2	5	6	14	14	cattle
MI10/07686	5	4	4	3	5	10	2	5	9	15A	15	pig
DICM08/01277-10B	5	4	4	3	5	10	2	7	9	15B	15	red deer
99/0281	5	4	3	3	5	5	2	6	5	16A	16	cattle
MI06/07005	5	4	3	3	4	5	2	5	5	16B	16	cattle
00/00178T	6	4	3	3	2	10	2	5	10	17A	17	cattle
MI05/00841	6	4	3	3	5	10	2	5	10	17B	17	cattle
MI07/06666	6	4	3	3	2	10	2	5	9	18A	18	cattle
MI09/04329	6	4	3	3	5	10	2	5	9	18B	18	wild boar
00/0197	1	3	3	3	5	10	2	5	8	19	19	cattle
MI06/05041	3	2	3	3	5	7	2	5	6	20	20	cattle
MI06/05481	3	3	3	3	5	10	2	5	9	21	21	cattle
MI07/12732	4	3	2	3	5	>15	2	5	7	22	22	cattle
MI07/11662	4	4	3	3	5	10	2	5	9	23	23	cattle
02/0440	5	3	3	3	5	10	2	5	10	24	24	wild boar
98/0144	5	3	3	3	5	10	2	5	6	25	25	cattle
MI07/08732	5	3	3	3	5	10	2	5	8	26	26	cattle
MI05/01528	5	3	3	3	5	9	2	5	7	27	27	cattle
98/0679	5	5	3	2	5	10	2	5	8	28	28	cattle
MI06/00126	5	4	3	3	5	10	2	5	15	29	29	cattle
99/0095	5	4	3	3	5	10	2	5	4	30	30	cattle
MI10/06535	5	4	3	3	5	10	3	6	6	31	31	pig

[a] Isolate reference.
[b] MIRU-VNTR loci with corresponding alias.
[c] MIRU-VNTR types obtained with the nine-loci approach.
[d] MIRU-VNTR types obtained with the four-loci approach. Table is arranged according to the number of isolates with each type in descending order.

ID[a]	MIRU-VNTR loci[b]									MIRU-VNTR types (9 loci)[c]	MIRU-VNTR types (4 loci)[d]	Animal origin
	ETR-A	ETR-B	ETR-D	ETR-E	MIRU26	QUB11a	QUB11b	QUB26	QUB3232short			
	2165	2461	580	3192	2996	2163a	2163b	4052	3232			
MI06/06077	5	4	3	3	5	7	2	5	8	32	32	cattle
MI05/04423	5	4	3	3	5	9	1	5	11	33	33	cattle
MI10/08243	5	4	3	3	5	9	2	5	8	34	34	fallow deer
MI08/00955	5	4	3	3	6	12	2	4	6	35	35	cattle
MI05/04048	5	4	4	3	5	10	2	5	5	36	36	cattle
MI06/02387	5	5	3	3	5	10	2	5	6	37	37	cattle
MI08/06294	5	6	3	3	5	10	2	5	11	38	38	cattle
99/0254	6	3	3	3	5	9	2	5	5	39	39	cattle
MI06/06841	6	4	2	2	5	10	2	4	5	40	40	cattle
MI07/03947	6	5	3	2	4	10	2	5	8	41	41	cattle
MI05/00703	6	4	3	3	5	10	2	5	11	42	42	cattle
MI07/08688	6	4	3	3	6	11	2	4	14	43	43	cattle
MI05/00050	6	4	3	3	6	12	2	4	12	44	44	wild boar
MI08/00951	6	4	3	3	6	12	2	4	13	45	45	cattle
98/0686	6	4	3	3	6	13	2	4	14	46	46	cattle
MI06/07218	6	5	3	2	5	10	2	5	9	47	47	cattle
MI06/07031	8	5	3	3	5	10	2	5	7	48	48	cattle
MI08/11986	6	4	3	3	4/5	4	N	2/5	7	-	49	badger
MI07/11175	6	4	3	N	5	8	2	4	8	-	50	cattle
MI08/02787	7	4	2	N	5	6	1	5	11	-	51	cattle
99/0098	5	4	3	3	5	N	2	5	4	-	-	cattle
MI05/04378	12	5	3	3	4	N	2	4	8	-	-	cattle
MI06/05469	N	3	3	3	5	10	2	5	10	-	-	cattle
MI07/00701	5	3	4	3	5	N	N	5	11	-	-	cattle
MI07/03936	4/5	3	3	N	5	10	2	5	8	-	-	cattle
MI07/04598	5	3/4	3	N	N	N	2	4/5	7	-	-	cattle
MI07/11661	5	4	3	3	5	N	2	5	5	-	-	cattle
MI10/05128	N	4	3	3	N	N	2	N	8	-	-	badger

[a] Isolate reference.
[b] MIRU-VNTR loci with corresponding alias.
[c] MIRU-VNTR types obtained with the nine-loci approach.
[d] MIRU-VNTR types obtained with the four-loci approach. Table is arranged according to the number of isolates with each type in descending order.

II.2 Limitations of spoligotyping and VNTR typing in a high diversity setting

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Limitations of Spoligotyping and Variable-Number Tandem-Repeat Typing for Molecular Tracing of *Mycobacterium bovis* in a High-Diversity Setting▽†

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This study describes the attempt to trace the first *Mycobacterium bovis* outbreak in alpacas (*Lama pacos*) in Spain by spoligotyping and variable-number tandem-repeat (VNTR) analysis. Due to high genotype diversity, no matching source was identified, but local expansion of a clonal group was found and its significance for molecular tracing is discussed.

Mycobacterium bovis is the etiological agent of bovine tuberculosis, but it can affect other domestic animals and wildlife. Molecular typing methods such as spoligotyping (17) and multilocus variable number tandem repeat (VNTR) analysis (10, 29) are wildly used to clarify epidemiological relations between outbreaks. Recently, three cases of *M. bovis* in alpacas (*Lama pacos*) have been reported as the first description of tuberculosis in this animal species in Spain (11). Since trade with camelids is increasing and scarce legislation addressing tuberculosis in these animals is given, a follow-up by molecular typing was deemed advisable to obtain insight into its epidemiology.

The alpacas had been held in two different, but epidemiologically linked herds: herd 1 (alpacas 1 and 2, with one animal imported from Peru and the other one from the United Kingdom) was located in the Ronda region (southern Spain), and herd 2 (alpaca 3, imported from Peru and moved from herd 1 to herd 2) was located 90 km away in the Antequera region (see Fig. S1 in the supplemental material). Spoligotyping (17) revealed an identical spoligotype profile, SB0295, for the three alpaca isolates (11). We excluded the possibility of infection in the country of origin in the case of the United Kingdom, because this spoligotype has not been reported (5, 14), and we ignore the situation in Peru due to the lack of molecular typing data from this country.

This study describes the findings of the attempt to trace back the outbreak by molecular characterization of *M. bovis* isolates from the greater surroundings of the alpaca farms.

In order to investigate the potential source of infection, a search of *M. bovis* spoligotypes, obtained according to the standard method (17), was performed in the Spanish Database of Animal Mycobacteriosis (http://www.vigilanciasanitaria.es/mycodb/index-en.php). For this purpose, we defined the geographical area within a radius of 150 km around the alpaca farms, considering Doñana National Park in the west, the Baetic Cordillera in the East, the Sierra Morena in the North, and the Mediterranean Sea in the South as natural geographical borders (see Fig. S1 in the supplemental material). In this study area, we gathered information from 171 cattle farms infected with *M. bovis*, two isolates from red deer (*Cervus elaphus*), and one from wild boar (*Sus scrofa*). These *M. bovis* isolates presented 36 different spoligotypes.

First, we considered that the most probable source could be an *M. bovis* isolate with a matching spoligotype (SB0295) obtained during the previous 5 years. This search retrieved 31 farms, and one isolate was randomly selected from each. Second, on the assumption that the evolution of the direct repeat (DR) locus is unidirectional by deletion of single or contiguous spacers (32), we also searched for related spoligotypes that could have led to or could have been derived from SB0295 and were present in the same farm or same municipality as *M. bovis* isolates with spoligotype SB0295. This search yielded 14 additional isolates with spoligotype SB0121 (which could generate SB0295 by loss of spacer 37) and one isolate with spoligotype SB1190 (which could derive from SB0295 by loss of spacer 2) (Table 1). In total, 44 cattle farms matched these criteria out of the 171 *M. bovis*-affected farms; also one isolate from red deer (SB0121) was found.

Mycobacterial interspersed repetitive-unit (MIRU)-VNTR typing was conducted on the selected 47 isolates with spoligotypes SB0295, SB0121, and SB1190 following the protocol described by Frothingham and Meeker-O'Connell (10), using nine VNTR markers: ETR-A (VNTR2165) (1, 26), ETR-B (VNTR2461) (10), ETR-D (MIRU4 [VNTR580]) (1, 26), ETR-E (MIRU31 [VNTR3192]), MIRU26 (VNTR2996) (28),

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TABLE 1. VNTR typing results from the *Mycobacterium bovis* isolates in this study

Identification no.[a]	Farm	Spoligotype[b]	MIRU-VNTR locus (VNTR no.)[c]									VNTR type
			ETR-A (2165)	ETR-B (2461)	ETR-D (580)	ETR-E (3192)	MIRU26 (2996)	QUB11a (2163a)	QUB11b (2163b)	QUB26 (4052)	QUB3232 (3232)	
MI05/00415	1	SB0295	6	4	3	3	5	11	2	5	7	A
MI05/04818	2	SB0295	6	4	3	3	5	11	2	5	8	B
MI05/04819	3	SB0295	6	4	3	3	5	11	2	5	7	A
MI05/04843	4	SB0295	6	2	3	3	5	11	2	5	7	C
MI06/05219	5	SB0295	6	4	3	4	5	11	2	5	7	D
MI06/05242	6	SB0295	6	4	3	3	5	12	2	5	7	E
MI06/05243	7	SB0295	6	4	3	3	5	12	2	5	7	E
MI07/10875	8	SB0295	6	4	3	3	5	11	2	5	7	A
MI07/11191	9	SB0295	6	4	3	3	5	11	2	5	7	A
MI07/13777	10	SB0295	6	4	3	3	5	11	2	5	8	B
MI08/01247	11	SB0295	4	4	3	3	5	11	2	5	13	F
MI08/06612	12	SB0295	6	4	3	3	5	11	2	5	7	A
MI08/08737	13	SB0295	6	4	3	3	5	11	2	5	8	B
MI08/09187	14	SB0295	6	4	3	3	5	11	2	5	7	A
MI08/10025	15	SB0295	6	4	3	3	5	11	2	5	7	A
MI08/13490	16	SB0295	6	4	3	3	5	11	2	5	12	G
MI08/13492	17	SB0295	6	4	3	3	5	11	2	5	12	G
MI08/13504	18	SB0295	6	4	3	3	5	11	2	5	7	A
MI09/00035	19	SB0295	3	3	3	3	5	11	2	4	7	H
MI09/02177	20	SB0295	6	4	—[d]	3	5	11	2	5	7	ND[e]
MI09/02887	21	SB0295	6	5	3	3	6	11	2	5	7	I
MI09/02890	22	SB0295	6	4	3	3	4	11	2	5	7	J
MI09/02897	23	SB0295	6	4	3	3	5	11	2	4	7	K
MI09/05468	24	SB0295	6	4	3	3	5	11	2	5	7	A
MI09/08242	25	SB0295	6	4	3	3	5	11	2	5	7	A
MI09/09006	26	SB0295	6	4	3	3	5	11	2	5	7	A
MI09/09018	27	SB0295	6	4	3	3	5	11	2	5	7	A
MI09/09022	28	SB0295	6	4	3	3	5	11	2	5	7	A
MI09/09027	29	SB0295	6	4	3	3	—	11	2	—	8	ND
MI09/09859	30	SB0295	6	4	3	3	5	11	2	5	7	A
MI09/10345	31	SB0295	6	4	3	3	5	11	2	5	7	A
MI09/08261	A1[f]	SB0295	6	4	3	4	5	11	2	5	6	L
MI09/09364	A1	SB0295	6	4	3	4	5	—	2	5	6	ND
MI09/10805	A2[f]	SB0295	6	4	3	4	5	11	2	5	6	L
MI05/04829	5	SB0121	6	2	3	3	5	11	1	5	6	M
MI05/04844	32	SB0121	5	4	3	3	5	11	2	5	9	N
MI06/05234	33	SB0121	5	4	3	3	5	11	2	5	9	N
MI07/01182	34	SB0121	6	4	3	3	5	11	2	5	7	A
MI08/01249	35	SB0121	6	4	3	3	2	11	2	5	9	O
MI08/06944	36	SB0121	5	4	3	3	5	11	2	5	8	P
MI08/09481	37	SB0121	6	4	3	3	5	11	2	5	7	A
MI08/09485	38	SB0121	4	4	3	3	5	9	2	5	9	Q
MI08/13503	39	SB0121	5	4	3	3	5	11	2	5	13	R
MI09/00032	40	SB0121	5	4	3	3	5	11	2	5	11	S
MI09/02889	41	SB0121	6	4	3	3	5	11	2	5	7	A
MI09/06628	42	SB0121	6	4	3	3	5	11	2	5	7	A
MI09/09232	Wildlife[g]	SB0121	5	4	3	3	5	10	2	5	8	T
MI09/09274	43	SB0121	5	4	3	3	5	11	2	5	8	P
MI09/10347	31	SB0121	5	4	3	3	5	10	2	5	8	T
MI07/11145	44	SB1190	6	4	3	3	5	11	2	5	7	A

[a] The isolates are arranged regarding the spoligotype and in ascending order according to the year of isolation. The first two digits after MI in the strain reference correspond to the year of isolation.

[b] International nomenclature according to Mbovis.org. ■ indicates the presence of a spacer, and □ indicates the absence of the spacer, as shown below.

SB0295: ■■□■■■■■■□■■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□■□□□□□

SB0121: ■■□■■■■■■□■■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□

SB1190: ■□□■■■■■■□■■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□

[c] Shown is the MIRU-VNTR locus with the corresponding VNTR number in parentheses.

[d] —, not amplifiable.

[e] Not determined because of the lack of at least one locus result.

[f] Alpaca farms (11).

[g] Isolate from red deer.

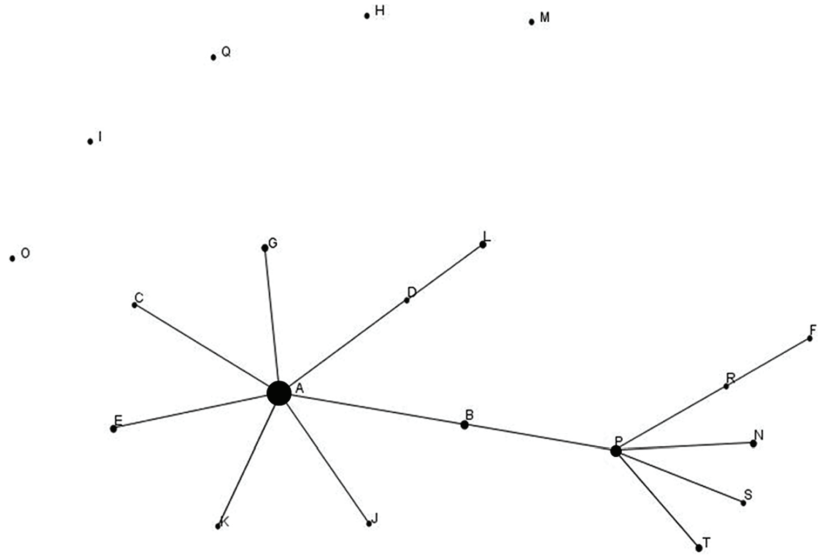

FIG. 1. Population snapshot of allelic profiles of the 20 VNTR types (A to T) using eBURST V3 (25). Groups were defined as sets of related isolates sharing identical alleles at eight of nine loci with at least one other member of the group. Type A is designated the putative founder of a clonal group and type P as the subgroup founder of a linked cluster. Five VNTR types (H, I, M, O, and Q) were not associated with any of the clonal groups.

QUB11a (VNTR2163a), QUB11b (VNTR2163b) (1, 26), QUB26 (VNTR4052) (23), and QUB3232 (VNTR3232) (26).

MIRU-VNTR typing of the cattle isolates SB0295 resulted in 11 different profiles (Table 1; see Fig. S1 in the supplemental material), but none of the profiles matched the profile of the alpaca isolates (VNTR type L). There was no variation at loci ETR-D and QUB11b. Locus QUB3232 was the most discriminatory locus, followed by ETR-B, which is consistent with other studies (1, 7, 9). Loci QUB3232 and QUB11a have been previously found to have a higher mutation rate regarding allelic diversity in *M. tuberculosis* and were therefore considered hypervariable (27); nevertheless, their use remains controversial as they may contribute to improved discrimination in determined settings (15). Conflicting findings may be due to variations between different lineages (31) or between species; e.g., allelic diversities may differ in *M. bovis* (18, 19). For the additional 16 isolates of different spoligotypes, QUB3232 was also the most discriminatory locus, followed by ETR-A and QUB11a. Loci ETR-B, MIRU26, and QUB11b achieved only poor discrimination, and loci ETR-D, MIRU31, and QUB26 did not vary at all. The index of discrimination (D) (16) was calculated using the *In Silico* website of the University of the Basque Country (http://www.insilico.ehu.es). The combination of spoligotyping and VNTR analysis yielded 22 different genotypes, and the discriminatory power was unexpectedly high ($D = 0.889$) given that isolates had been selected according to previously mentioned criteria.

A population snapshot for the VNTR typing results (A to T) was performed by eBURST V3 (25), which has been suggested for analyzing populations with low or moderate recombination ratios (30), such as *M. bovis* (24). This analysis revealed a clonal group and a linked cluster consisting of related isolates sharing identical alleles at eight out of the nine loci with at least one other member of the group (Fig. 1). VNTR type A (6-4-3-3-5-11-2-5-7) was the most frequent type and was designated the putative founder. It was present in 20 farms and has been found throughout the years and all over the region. VNTR type P was the putative subgroup founder of the linked cluster. The software did not associate five of the VNTR types (H, I, M, O, and Q) with any group due to variations at more than one locus without link to any of the patterns. These findings could be compatible with expansion of a frequent pattern (VNTR type A) from which single-locus variants (SLVs) are derived. Such SLVs could be signs of genetic drift in clonal subpopulations of *M. bovis* (2, 22).

Relationships between the isolates using combined results of spoligotyping and MIRU-VNTR typing were calculated using the MIRU-VNTRplus tool to create an unweighted-pair group method with arithmetic mean (UPGMA) tree with default parameters (see Fig. S2 in the supplemental material). Consistent with eBURST analysis, the largest group clustered isolates with VNTR type A consisting of three subgroups of different spoligotypes (SB0295, SB0121, and SB1190). Due to the fact that SB0121 is ancestral to SB0295, which is derived by a single deletion step in the DR region, we hypothesize that an *M. bovis* strain with spoligotype SB0121 and VNTR type A is the putative founder of the main cluster.

According to a recent study, SB0295 is the fifth most frequent spoligotype in Spain, representing 4.1% of isolates (21). However, its prevalence is highest in the Autonomous Community Andalusia (18.7%). It has also been found in France (13) and Portugal (8). In Spain, *M. bovis* spoligotype SB0295

has been mainly isolated from cattle (96% of isolates), and it has also caused infection in deer, wild boars, and Iberian lynxes (3, 12, 22). Although no local wildlife isolates of spoligotype SB0295 or related spoligotypes were registered in the national database, we do not disregard the possibility of wildlife acting as a source of infection. In the Mediterranean habitats of central and southern Spain, wild boars and other ungulates are overabundant, with only little fencing being practiced (4, 20). Nowadays, cattle farms undergo strict control measures; however, extensive herd management is still widespread in Spain, and wildlife is known to intrude into livestock habitats despite fencing.

This study shows the importance of exhaustive collection of molecular typing data of both domestic and wildlife isolates for successful tracing. Ongoing outbreaks with continued transmission are more likely to show genetic diversity than are point source outbreaks (6); therefore, typing results also depend on the nature of the outbreak under investigation. Facing a high-diversity scenario, a less stringent interpretation of typing results may be necessary. Although we could not identify an identical genotype in this attempt to trace back the *M. bovis* outbreak in the alpacas, we suggest taking clonal groups of closely related strains into account when possible sources of infection need to be elucidated, especially in high-diversity settings.

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Fig. S1. Map of the region from which the *Mycobacterium bovis* isolates were obtained showing the distribution of genotypes A-V obtained by combination of spoligotypes (SB0295, SB0121 and SB1190) and variable number tandem repeat profiles (A-T) according to cattle farms (1-44), red deer (D1) and alpaca farms (A1, A2).

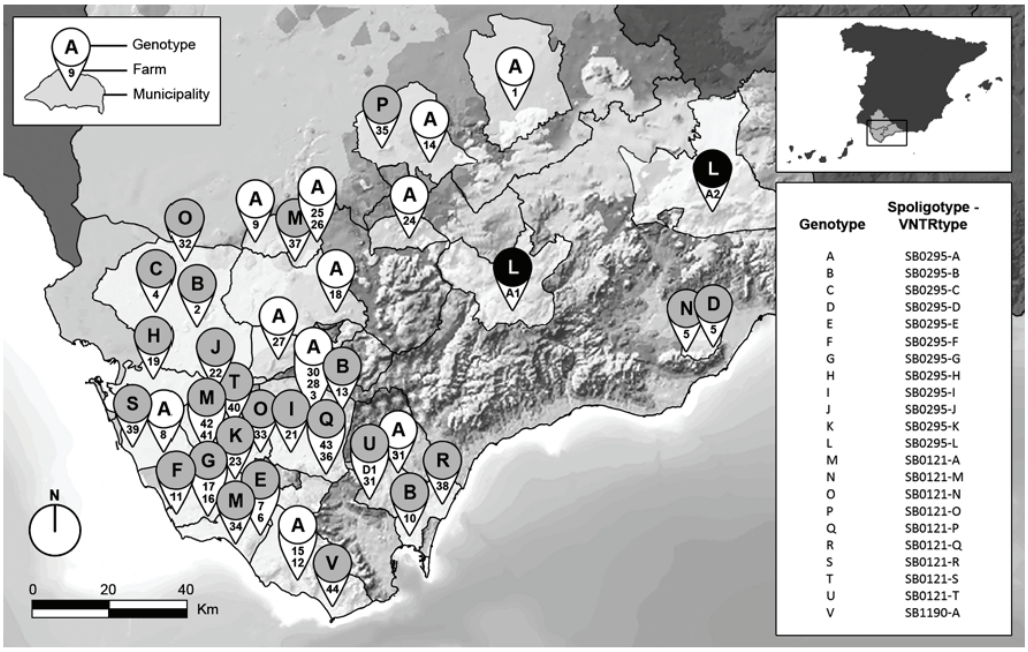
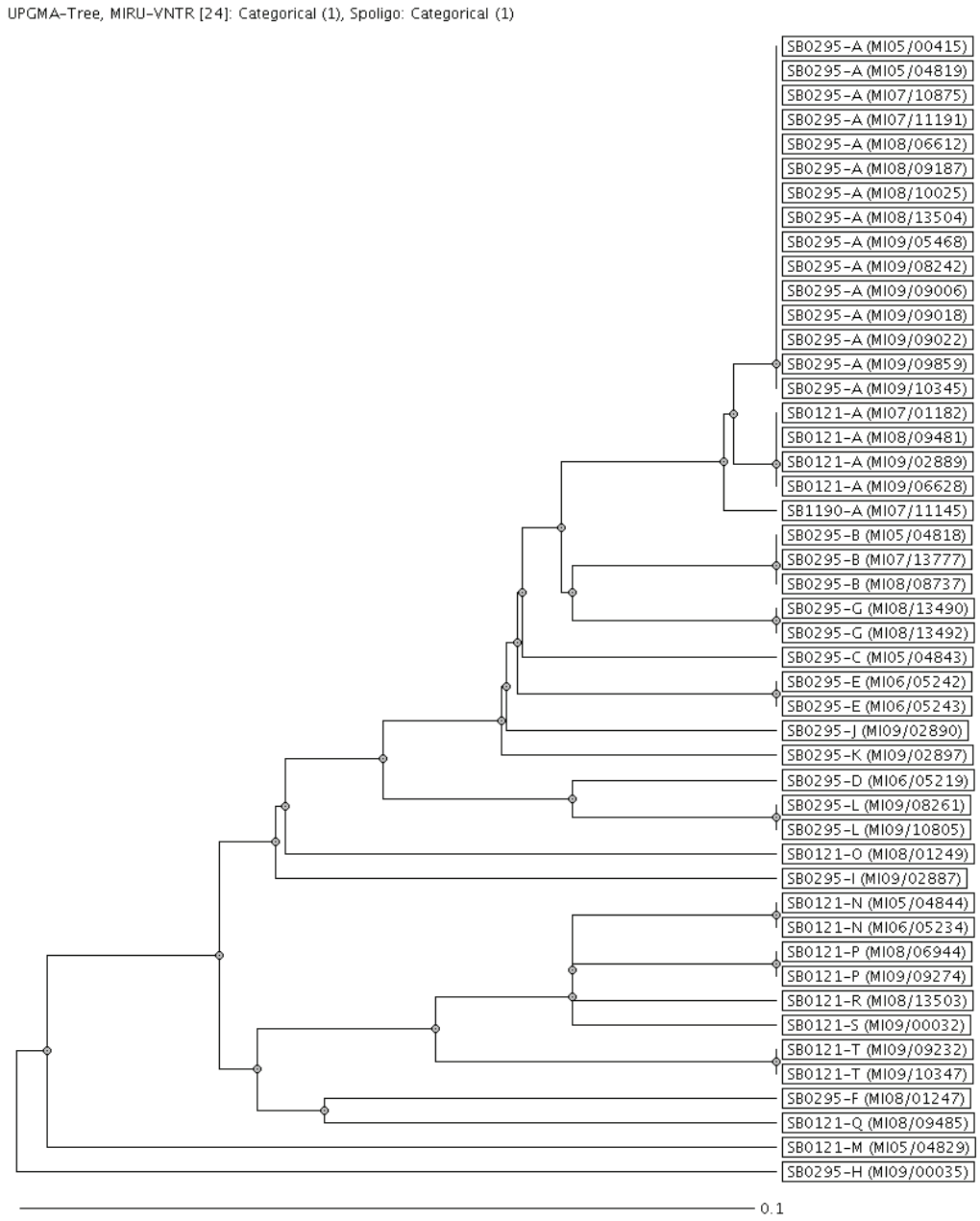


Fig. S2. Dendrogram for 47 *Mycobacterium bovis* isolates (not determined types were excluded) based on combined results of spoligotyping and variable number tandem repeat analysis using the UPGMA tool on the MIRU-VNTRplus website (2) with default parameters. Loci QUB11a and QUB3232 were arbitrarily assigned to database fields of standard loci (QUB5 and QUB4156).



II.2.1 Tuberculosis in alpacas caused by *M. bovis*

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Tuberculosis in Alpacas (*Lama pacos*) Caused by *Mycobacterium bovis*▽

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We report three cases of tuberculosis in alpacas from Spain caused by *Mycobacterium bovis*. The animals revealed two different lesional patterns. Mycobacterial culture and PCR assay yielded positive results for *M. bovis*. Molecular typing of the isolates identified spoligotype SB0295 and identical variable-number tandem repeat (VNTR) allele sizes.

CASE REPORT

Herd 1. The affected herd comprised 32 alpacas (26 adults and six juveniles) and was located in the Ronda region (southern Spain). The animals were raised in outdoor facilities and were fed with commercial pellet feed, hay, and water *ad libitum*. In March 2009, a 7-year-old female alpaca (alpaca 1) showed dyspnea, fever, depression, lethargy, anorexia, and weight loss. One month later, an 8-year-old male alpaca (alpaca 2) presented chronic weight loss, bruxism, and dyspnea.

Herd 2. The second herd was located in the Antequera region (southern Spain; approximately 90 km away from herd 1) and comprised four animals reared under intensive conditions. In July 2009, a 3-year-old female alpaca (alpaca 3) showed appetite loss, recumbency, muscle weakness, dyspnea, and bruxism. This animal had been moved from herd 1 6 months before and gave birth to a healthy male cria (alpaca 4) in December 2008. Alpaca 4 died 2 months after alpaca 3 with clinical signs associated with an enteric disorder.

Alpacas 1, 2 and 3 were subjected to X-ray analysis. Alpacas 1 and 2 showed cavitary lesions with heterogeneous areas of increased density inside and severe and diffuse consolidation of pseudonodular morphology in lung parenchyma. On the other hand, diffuse consolidation with micronodular and interstitial infiltrate was observed in alpaca 3. Ultrasonographic examination revealed a hypoecogenic area with hyperecogenic areas of various sizes above the heart and parenchyma within the liver and the spleen (alpacas 1 and 2). The three animals were treated with a combination of potassium penicillin (20,000 IU/kg of body weight, administered by intramuscular injection [IM], every 12 h for 10 days), gentamicin (6.6 mg/kg, IM, every 24 h for 5 days), flunixin meglumine (0.25 mg/kg, IM, every 12 h for 10 days), and metronidazole (10 mg/kg, IM, every 12 h for 10 days). No clinical improvement was observed in any case.

One month before the first case was detected, a comparative intradermal tuberculin test (IDT) (bovine and avian tuberculins from CZV, Porriño, Spain) and gamma interferon (IFN-γ) test (Bovigam test; Prionics AG, Schlieren, Switzerland) were conducted with herd 1 by the Official Veterinary Services. All alpacas were negative to both tests. However, due to the suspicion of tuberculosis (TB) infection in alpaca 1, a program of repeated intradermal tests at 90-day intervals was again implemented for herd 1 in late April 2009. Blood samples for serological testing were also taken both on the day of tuberculin injection and 21 days later. On the other hand, 1 week after the first clinical signs were observed in alpaca 3, the same program was carried out in herd 2 in late July 2009. The analyses yielded negative results in both herds.

In the three alpacas (alpacas 1, 2, and 3), the disease was progressive, and despite treatment under veterinary supervision, the animals were finally euthanized and submitted to the Veterinary Medicine Faculty of the University of Cordoba at the owner's request. Postmortem examination of alpacas 1 and 2 revealed multifocal-to-coalescing, granulomatous, caseous, and calcified nodules of different diameters (from 2 mm to 10 cm) in lung, trachea, liver, and spleen and in tracheobronchial, mediastinal and mesenteric lymph nodes. At the cut sections, these nodules were yellowish and firm with a partially mineralized core. In addition to the nodular pattern, the lungs showed a diffuse pattern as well as lesions which communicated with the lumens of bronchi (Fig. 1, inset). These lesions together with the ulcerative, granulomatous lesions observed in the mucosa of the trachea are considered indicative of open TB. Alpaca 3 showed multifocal-to-coalescing, miliar, granulomatous pneumonia, pleuritis, and peritonitis covering the thoracic and abdominal cavities (miliary TB). The death of alpaca 4 was associated with an intussusception process. No gross lesions compatible with TB were observed in this animal. At the necropsy of each animal, samples from selected organs were

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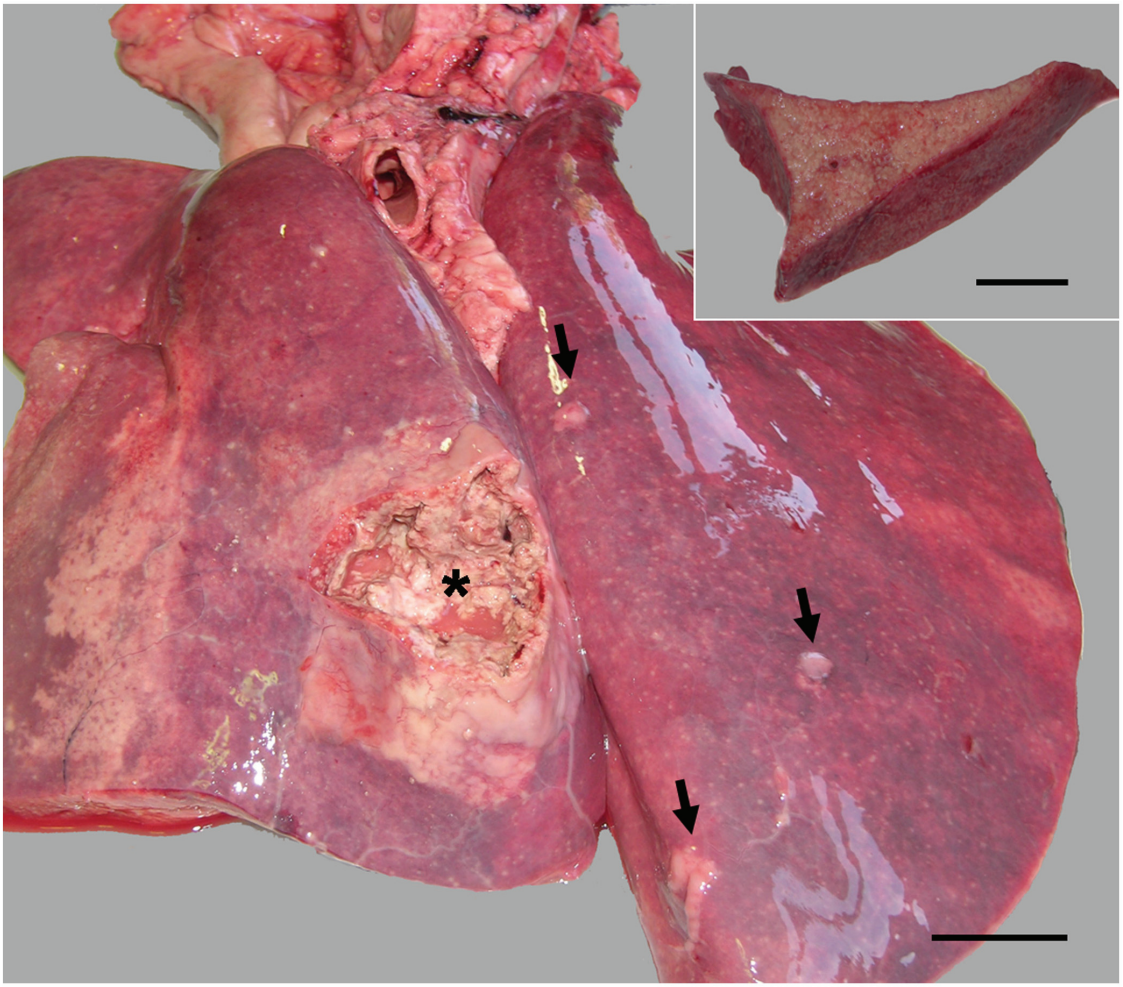

FIG. 1. Multifocal areas of granulomatous, caseous bronchopneumonia (arrows) with a large area of bronchopneumonia communicating with the bronchial lumen (asterisk). Bar = 5 cm. The inset image shows a diffuse pattern of granulomatous pneumonia observed in alpaca 1. Bar = 4.5 cm.

collected for histopathological, bacteriological, and molecular studies.

Tissue samples of different organs were fixed in 10% neutral buffered formalin, routinely processed, and stained with hematoxylin and eosin and Ziehl-Neelsen staining. Histopathology revealed granulomatous lesions composed of a central core of epithelioid macrophages and scattered lymphocytes and plasma cells at the periphery. Epithelioid macrophages showed a finely granular, foamy cytoplasm. Some granulomatous lesions showed a central core of necrosis and occasional mineralization (Fig. 2A). Granulomata were surrounded by a marked connective tissue capsule. In addition to the nodular pattern, alpacas 1 and 2 showed a diffuse pattern of granulomatous inflammation with lack of delimiting capsule in lung and trachea (Fig. 2C), which focally ulcerated the epithelium of the mucosa of bronchi and trachea. Abundant acid-fast bacteria (AFB) were identified on Ziehl-Neelsen-stained smears and tissue sections of all the tissues examined in alpacas 1 and 2 (Fig. 2B and C, inset), whereas alpaca 3 showed a paucibacillary staining with mycobacteria being identified only occasionally.

A pool of lung, spleen, liver, and tracheobronchial lymph node homogenates was subjected to specific mycobacterial culture by using standard procedures. The homogenates, previously decontaminated with hexadecylpyridinium chloride, were cultured on Löwenstein-Jensen medium with pyruvate and Coletsos solid selective media (Biomedics, Madrid, Spain). Growth of mycobacteria in the specific culture was obtained in the three alpacas. Identification of *Mycobacterium bovis* (alpacas 1, 2, 3) was performed from suspected colonies by using a multiplex PCR amplification of the fragments coding

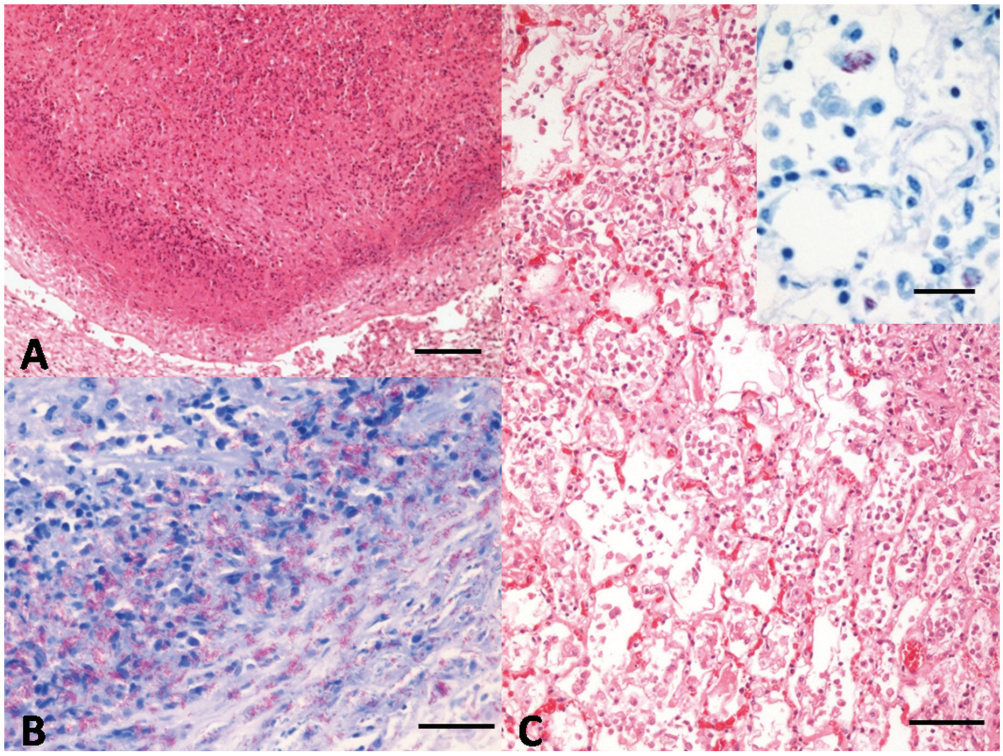

FIG. 2. (A) Alpaca 1. Nodular granulomatous lesion composed of a central core of necrosis, surrounded by degenerated neutrophils and cell debris, epithelioid macrophages, and scattered lymphocytes and plasma cells. Note the manifest capsule of connective tissue delimiting the lesion (as shown by hematoxylin and eosin [H&E] staining). Bar = 100 μm. (B) Alpaca 1. Abundant acid-fast bacteria in the periphery of the granulomatous lesion. Ziehl-Neelsen staining. Bar = 20 μm. (C) Alpaca 3. Marked proliferation of histiocytes, with diffuse intraalveolar infiltrate of foamy macrophages and epithelioid cells. H-E. Bar = 50 μm. The inset image shows random foamy macrophages, laden with numerous acid-fast bacteria within their cytoplasm. Ziehl-Neelsen staining. Bar = 20 μm.

for rRNA 16S and MPB70 protein (24). Moreover, *Mycobacterium tuberculosis* complex (MTC) DNA was also directly identified from fresh tissue samples by PCR based on an MTC-specific IS*6110* insertion sequence (13). Spoligotype SB0295 was identified using the standardized membrane with 43 spacers as previously described (12). Variable-number tandem repeat (VNTR) typing was carried out as described by Frothingham and Meeker-O'Connell (9) by using nine VNTR markers (ETR-A [VNTR2165], ETR-B [VNTR2461], ETR-D [VNTR580], ETR-E [VNTR3192, MIRU31], MIRU26 [VNTR2996], QUB11a [VNTR2163a], QUB11b [VNTR2163b], QUB26 [VNTR4052], QUB3232 [VNTR3232]). The VNTR profiles were identical for all isolates: 6-4-3-4-5-11-2-5-6 (order of markers as described above).

Alpaca 4 showed negative results to both PCR and specific mycobacterial culture, and *Clostridium perfringens* was isolated in large quantities in the digestive tract.

TB is an infectious disease responsible for millions of human deaths annually and significant economic losses in livestock worldwide (5, 19). In many countries, *M. tuberculosis* and *M. bovis* are the most common agents isolated in TB cases in humans and ruminant species, respectively (7). These pathogens that belong to MTC affect also a wide range of domestic and wild species (7, 15). The disease in South American camelids has recently acquired importance since alpacas and llamas are being imported and kept in increasing numbers in many European countries (2). Camelids are known to be susceptible to MTC, including *M. tuberculosis*, *M. bovis*, and/or *Mycobacterium microti* (8, 17, 23), and to *Mycobacterium kansasii* infections (11). Furthermore, TB cases have been recently reported in alpacas and llamas from different European countries (2, 14, 16, 20).

Although *M. bovis* was isolated in the three alpacas, two different lesional patterns were observed. Alpacas 1 and 2 showed a combination of both nodular and diffuse patterns of TB in lungs and trachea together with ulceration of the mucosa and numerous AFB. Similar lesions have been previously reported in alpacas, other camelid species, and wild ruminants (3, 14, 16, 21, 22). On the other side, alpaca 3, which was also infected by *M. bovis*, showed miliary TB lining the pleural and

peritoneal cavities with scarce AFB. The lesional pattern found in this animal was similar to those observed in cattle (3). The diffuse pattern in contrast to the nodular pattern may point to a failure in the control of the lesion, with the host immune response not able to delimit and isolate the affected from the nonaffected parenchyma by a connective tissue capsule. Moreover, the evidence of open TB in trachea and lung suggests that this animal species may be a potential source of mycobacterial excretion.

The antemortem detection of TB in camelids presents many difficulties, with none of the currently available tests being able to detect disease with certainty (22). In our study, neither comparative IDT nor IFN-γ tests were able to identify the positive animals in the herds. The Bovigam test is a current method of diagnosis for cattle; however, it has already been reported to be a nonvalid test for the diagnosis of TB in camelids (22). The intradermal tuberculin test, which is the traditional diagnostic approach for a number of other species, is believed to produce nonspecific reactions in camelids (6, 8, 20). Serological assays may be a promising alternative, but little is known about antibody responses during TB in these species (14, 23). There have been previous attempts to develop alternative immunodiagnostic assays for TB in camelids based on *in vitro* lymphocyte transformation or antibody measuring by enzyme-linked immunosorbent assay (ELISA) (10), but no reliable test is currently available. Furthermore, there is little evidence that detection of specific antibodies (using methods such as ELISA) could be a useful indicator of field infection (4). Recently, multiantigen print immunoassay (MAPIA) and lateral-flow-based rapid test (RT) have been experimentally showed as useful diagnostic tools for antemortem detection of TB in multiple host species, including camelids (6, 14, 23).

Although this is the first record of bovine TB in alpacas from Spain, the animals affected in the present study came from Peru (alpacas 1 and 3), the United Kingdom (alpaca 2), and Spain (alpaca 4). The *M. bovis* isolates were confirmed as spoligotype SB0295 with the VNTR profile 6-4-3-4-5-11-2-6-6 (ETR-A, ETR-B, ETR-D, ETR-E, MIRU26, QUB11a, QUB11b, QUB26, QUB3232). Spoligotype SB0295 represents 4.1% of the strains isolated from TB cases in domestic and wildlife species in Spain (1, 18). This spoligotype has been frequently isolated in cattle (94.1%) from southern regions (40.2%) in this country. This finding indicates that the animals were probably infected in Spain. In addition, the MIRU/VNTR typing also revealed identical profiles in the three affected alpacas. Therefore, alpaca 3 was probably infected in herd 1. Further molecular studies involving neighboring farms and wildlife are in progress in order to trace back the infection. In Spanish Mediterranean ecosystems, wildlife species are able to maintain *M. bovis* infection in the environment in the absence of domestic livestock and are probably able to transmit the disease to other species, acting as reservoirs (1, 15).

Transmission between alpacas by direct contact has been recently suggested (21). However, although alpaca 4 remained together with alpaca 3 all the time, *M. bovis* transmission by direct contact or via infected milk was not detected in this animal.

The results confirm the susceptibility of alpacas to *M. bovis* infection and show a wide variety of consequent pathological findings. The open TB observed in alpacas 1 and 2 suggests that this species may act as a potential source of mycobacterial excretion. Therefore, given the risk of transmission, not only to other domestic or wild species but also to human beings, the infection by *M. bovis* should be considered in the differential diagnoses of respiratory diseases in alpacas (8), particularly in recognized regions where TB is endemic. Moreover, our study highlights the difficulty of antemortem diagnosis using the official tests currently available for the diagnosis of TB in other species. In this sense, the use of complementary immunological diagnostic methods, such as RT and MAPIA, may provide a useful screening tool to identify infected animals (6, 14, 23).

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II.3 Spoligotyping and MIRU-VNTR typing of *M. bovis* isolates from bullfighting cattle

Bullfighting has a long tradition in Spain, Portugal, and southern France and in the South American countries Mexico, Colombia, Venezuela, Peru and Ecuador. The taurine breeds used for this purpose have special traits that distinguish them from the other breeds of domestic cattle. According to the Spanish *Real Decreto* (Royal Decree) 601/2001, 26 January, the Spanish bullfighting breeds date back to the medieval age and are characterised by their aggressiveness and resistance to any form of conventional management. Bullfighting cattle is held in purely extensive management in order to play to its capacity of adaptation and exploitation of natural resources in any ecosystem, and to favour their territorial behaviour. Although bullfighting cattle have a peaceful temperament in their natural habitat, handling of these animals is very difficult, sometimes even impossible, which hampers the procedures within eradication programmes, for example the skin test for bovine tuberculosis. Therefore the legislation in Spain has addressed bullfighting cattle separately in terms of animal health requirements in the *Real Decreto* 1939/2004, 27 September, which has recently been replaced by the stricter *Real Decreto* 186/2011, 18 February. This law regulates the sanitary qualification of bullfighting herds and lays down the requirements for the movement of bullfighting cattle. The prevalence of bovine tuberculosis in bullfighting herds is estimated to be high, but exact numbers are unknown (MARM 2010).

In order to gain a first insight into the epidemiological situation we used a molecular approach by spoligotyping and mycobacterial interspersed-variable number tandem repeat (MIRU-VNTR) typing of 39 isolates of *Mycobacterium bovis* from 16 bullfighting cattle farms.

The farms under study were located in the three main areas of bullfighting cattle breeding in central and southwest Spain, named *Zona Centro*, *Zona Mediodía* and *Zona Salamanca* (Figure 20A) (Unión de Criadores de Toros de Lidia, 2010) which belong to some of the veterinary districts with highest herd prevalence of bovine tuberculosis in Spain (Figure 20B) (MARM, 2011). The number of analysed isolates per farm oscillated between 1 and 10 (Table 10); five isolates originated from wildlife species (wild boar, n=3; red deer, n=1) living on the same premises with bullfighting cattle.

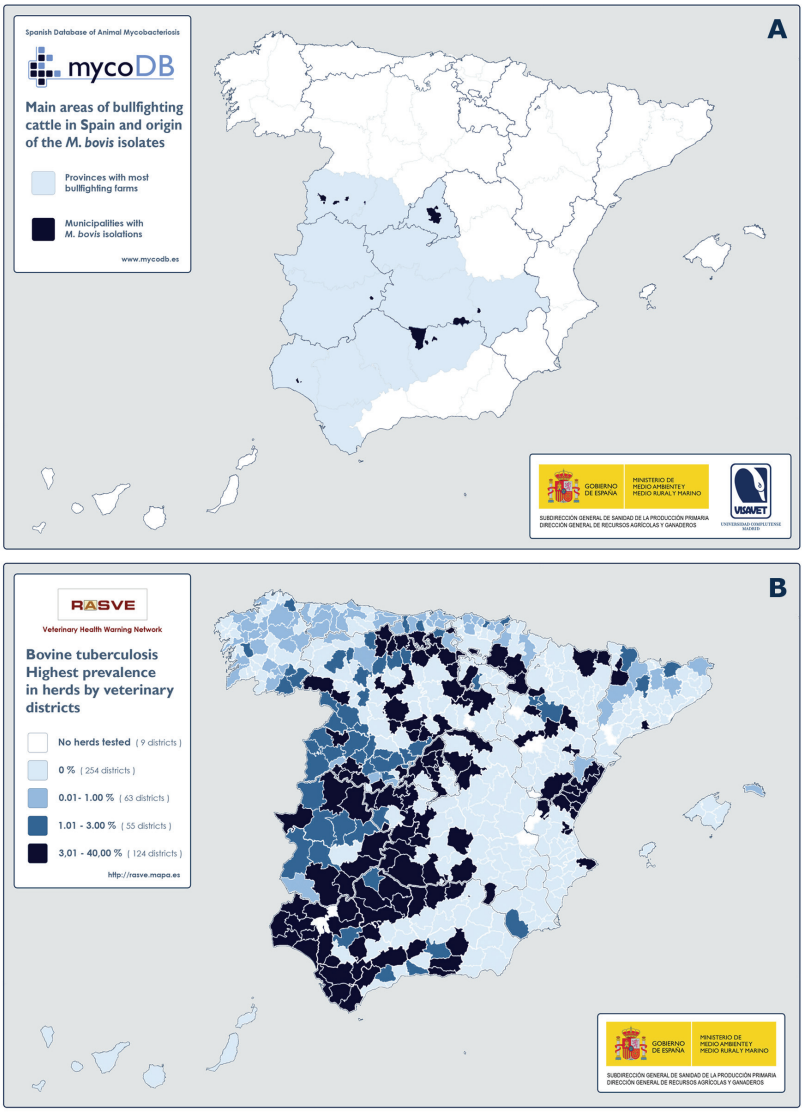

Figura 20. Maps of Spain showing (A) the provinces with most bullfighting farms (grey) and the municipalities of the farms included in this study (blue), and (B) the herd prevalence of bovine tuberculosis by veterinary district (MARM, 2011).

Spoligotyping was carried out following the standard protocol (Kamerbeek *et al.*, 1997) and resulted in 14 different patterns. For MIRU-VNTR typing (Frothingham and Meeker-O'Connell, 1998) the following nine MIRU-VNTR markers were applied: ETR-A (VNTR2165), ETR-D (MIRU4, VNTR580), QUB11a (VNTR2163a), QUB11b (VNTR2163b) (Allix *et al.*, 2006; Supply, 2006), ETR-B (VNTR2461) (Frothingham and Meeker-O'Connell, 1998), ETR-E (MIRU31, VNTR3192), MIRU26 (VNTR2996) (Supply *et al.*, 2001), QUB26 (VNTR4052) (Skuce *et al.*, 2002) and QUB3232 (VNTR3232) (Supply, 2006). With these nine loci the 39 isolates clustered in 21 different MIRU-VNTR types, while a reduction of the number of markers to four loci (ETR-A, ETR-B, QUB1a and QUB3232) resulted in 15 MIRU-VNTR types. The indices of discrimination (D) (Hunter and Gaston, 1988; Hunter, 1990) were calculated for each typing technique and different combinations of these, as well as for the individual loci (Table 9); for this purpose we used the in-silico website of

the University of the Basque Country (http://www.insilico.ehu.es) filling in the number of unrelated isolates sharing the same genotype. The highest discriminatory power is obtained by combining spoligotyping and 9-loci MIRU-VNTR typing (D=0.9577).

Table 9. Allelic diversity of the individual mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) markers and discriminatory indices (D) of spoligotyping and MIRU-VNTR typing and different combinations of these for the set of 39 isolates of *Mycobacterium bovis* from bullfighting cattle.

MIRU-VNTR locus (alias)	No. of isolates with MIRU-VNTR allele:														D
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	>15	N[a]	
3232 (QUB3232)					3	4	26	4	2								0.7661
2165 (ETR-A)	2		2	4	7	20	4										0.6964
2163a (QUB11a)							4		1	31	1	1			1		0.6209
2163b (QUB11b)	1	30	4	3	1												0.6144
2996 (MIRU26)		1		6	27	4											0.5947
2461 (ETR-B)		1		32	6												0.5417
4052 (QUB26)		1		7	31												0.5221
580 (ETR-D)			36	2												1	0.1429
3192 (ETR-E)			39														0.0000
spoligotyping																	0.9138
9 loci[b]																	0.9544
4 loci[c]																	0.9275
9 loci + spoligotyping																	0.9577
4 loci + spoligotyping																	0.9497

[a] Not amplifiable.
[b] Combination of ETR-A, ETR-B, ETR-D, ETR-E, MIRU 26, QUB11a, QUB11b, QUB26 and QUB3232.
[c] Combination of ETR-A, ETR-B, QUB11a and QUB3232.

The five wildlife isolates, which originated from animals hunted on bullfighting farms, showed the same genotypes as the cattle isolates in three cases and identical spoligotype combined with a similar MIRU-VNTR type (single locus variant) in

two cases. The most prevalent spoligotype among the selected *M. bovis* isolates was SB0295 (n=13; 33%); the 13 isolates included two isolates from wild boar and one isolate from red deer and originated from eight different farms, three of them with confirmed epidemiological links. SB0295 is the most prevalent spoligotype in southern Spain and also one of the most prevalent types in the centre of the country. Seven of the identified spoligotypes circulate in the greater (province) or close (municipality) surroundings of the farms and were in some cases also found in wildlife in the area (Table 10). In fact, the spoligotypes are among the fifteen most frequent spoligotypes in Spain, except from SB0833, SB0933, SB1310, SB1313, SB1321, SB1346 and SB1608, that have only been isolated once and could have evolved from the more prevalent spoligotypes by deletion events such as loss of one spacer or a block of contiguous spacers. Interestingly, the SB0295 isolates showed the same MIRU-VNTR type (6-4-3-3-5-10-2-5-7, in the following order: ETR-A, ETR-B, ETR-D, ETR-E, MIRU26, QUB11a, QUB11b, QUB26 and QUB3232), apart from two isolates with a single allele variation at MIRU26 (6 repeats) and double allele variation at loci MIRU26 and QUB11a (4 and 11 repeats, respectively). However, these isolates were epidemiologically linked to five isolates with the most frequent MIRU-VNTR type. This contrasts with the findings of a clonal group of closely related isolates with spoligotype SB0295 and different MIRU-VNTR types described in chapter II.2.

These results indicate that the infection in bullfighting cattle might be transmitted within the cattle or through wildlife present in the same geographical area.

Table 10. Presence of spoligotypes from other domestic or wild animal species in the same municipality or province of the 16 farms with bullfighting cattle.

Province	Municipality	Farm	No. of isolates[a]	Spoligotypes[b]	Present in municipality	Present in province	Wildlife[c]
Albacete	Povedilla	1	6 (3[d])	SB0121	-	✓	-
				SB0295	✓	✓	WB, RD
				SB0152	-	-	-
				SB0933	-	-	-
Cáceres	Cáceres	2	1	SB0265	✓	✓	WB
Ciudad Real	Alcoba	3	1	SB0295	-	✓	WB, RD
	Piedrabuena	4	1	SB0134	-	✓	WB, RD, B
	Villamanrique	5	3	SB0121	✓	✓	WB
				SB0265	✓	✓	WB, RD
				SB0295	-	✓	WB, RD
Huelva	San Juan del Puerto	6	1	SB0134	-	✓	-
Jaén	Andújar	7	(2[e])	SB0121	✓	✓	WB, RD
				SB0295	✓	✓	WB
		8	1	SB0295	✓	✓	WB
	Guarromán	9	1	SB0295	-	✓	WB
Madrid	Madrid	10	1	SB0121	✓	✓	WB, RD, FD
		11	1	SB0121	✓	✓	WB, RD, FD
		12	1	SB0295	✓	✓	WB
Salamanca	Chagarcia-Medianero	13	10	SB0120	-	✓	-
				SB0121	✓	✓	WB
				SB0295	✓	✓	-
				SB0339	-	✓	-
				SB1313	-	✓	-
				SB1321	-	-	-
				SB1608	-	-	-
	Las Veguillas	14	4	SB0121	✓	✓	WB
				SB0152	-	✓	-
				SB1310	-	-	-
	Sancti-Spíritus	15	1	SB1346	-	-	-
	Tamames	16	1	SB0833	-	✓	-

[a] Number of isolates from each farm included in the study. In parentheses the number of wildlife isolates from the same farm.
[b] International nomenclature according to www.mbovis.org.
[c] Same spoligotype found in wildlife species in the same province (WB: wild boar, RD: red deer, FD: fallow deer, B: badger).
[d] Wild boar, n=2; red deer, n=1.
[e] Wild boar, n=2.

II.4 Contributions to conferences and meetings of European projects

Rodríguez S., Castellanos E., de Juan L., Bezos J., Gallardo F., Moya N., Álvarez J., Álvarez N., Alende T., Gutiérrez A., Lozano F.J., Mateos A. and Romero B. Mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) typing of SB0121, the most frequent spoligotype in Spain. Oral presentation. Workshop "VNTR/MIRU and DVR-spoligotyping for *Mycobacterium bovis* typing. Results of the VNTR-DVR Spoligotyping Ring Trial". of European project SSPE-CT-2004-501903. Madrid (Spain), 24-25 March 2009.

Rodríguez S., Aranaz A., Bezos J., Castellanos E., de Juan L., Gallardo F., Gutiérrez A., Mateos A., Domínguez L. and Romero B. High discrimination of the MIRU-VNTR technique for the most frequent spoligotype in Spain. Oral presentation. *M. bovis* V Conference. Wellington (New Zealand), 25-28 August 2009.

Rodríguez S. Advances in Workpackage 6: Molecular characterisation of *M. bovis* and *M. caprae* isolates focused on epidemiological investigation. Oral presentation. Mid-term meeting of European project FP7-KBBE-2007-212414. Madrid (Spain). 11-12 November 2010.

Mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing of SB0121, the most frequent spoligotype in Spain

Rodríguez S., Castellanos E., de Juan L., Bezos J., Gallardo F., Moya N., Álvarez N., Alende T., Gutiérrez A., Lozano F., Mateos A. and Romero B.

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Among Spanish *Mycobacterium* (*M.*) *bovis* isolates the most prevalent spoligotype pattern is SB0121, which contains all *M. bovis* spacers except number 21. This spoligotype accounts for 27.9% of the *M. bovis* isolates and has been found throughout the country over the last 16 years. Furthermore, this spoligotype, which represents almost 30% of the cattle isolates, is also detected in goats and wildlife (red deer, fallow deer and wild boar). Other Mediterranean countries such as France, Portugal and Italy also described the isolation of SB0121, as well as reports from South Africa and South America. In contrast, no SB0121 isolates were reported from the British Isles.

In order to determine whether the high prevalence of this spoligotype can be due to incapacity of the DVR-spoligotyping to further discriminate these strains, we analysed a representative panel of SB0121 isolates (n=100) by MIRU-VNTR typing. A group of six loci was used to subtype the panel: ETR-A (VNTR2165), ETR-B (VNTR2461), ETR-D (VNTR580), QUB11b (VNTR2163b), QUB3232 (VNTR3232, short fragment) and QUB26 (VNTR4052). Five of the loci were included in the Multilocus Variable Number Tandem Repeat Genotyping of *M. bovis* Ring Trial VENoMYC 2006, and QUB26 was chosen because by our experience it provides a better discrimination than QUB11a (VNTR2163a). A differentiation into 55 profiles was achieved, with a discriminatory power (D) of 0.97. The three most discriminative loci in decreasing order were: VNTR3232, ETR-A and ETR-B.

This set of loci resulted highly discriminative for the Spanish *M. bovis* SB0121 spoligotype and therefore indicates the lack of discriminatory power of DVR-spoligotyping in this particular case.

High discrimination of MIRU-VNTR technique for the most frequent spoligotype in Spain (SB0121)

Rodríguez S.[1,2], Aranaz A.[1,2], Bezos J.[1,2], Castellanos E.[1,2], de Juan L.[1,2], Gallardo F.[1], Lozano F.[1], Mateos A.[1,2], Domínguez L.[1,2] and Romero B.[1,2]

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Spoligotyping and Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats (MIRU-VNTR) have been recognized as valuable genotyping techniques for *Mycobacterium* (*M.*) *bovis* and *M. caprae*. Among Spanish *M. bovis* isolates the most prevalent spoligotype pattern is SB0121, which contains all *M. bovis* spacers except number 21. This spoligotype accounts for 27.9% of the *M. bovis* strains and has been found throughout the country over the last 16 years. Although this spoligotype has been mainly isolated from cattle, it has also been detected in goats and wildlife (red deer, fallow deer and wild boar).

In order to assess whether MIRU-VNTR typing discriminates within this group, a representative panel of SB0121 strains (n=100), isolated from domestic and wild animals, and from several regions of Spain, was analyzed by this method. To subtype the panel a selection of six loci was used: ETR-A (VNTR2165), ETR-B (VNTR2461), ETR-D (VNTR580), QUB11b (VNTR2163b), QUB3232 (VNTR3232, short fragment) and QUB26 (VNTR4052). These are some of the most discriminatory loci used in previous studies (1, 2) and were also included in the MIRU-VNTR Genotyping of *M. bovis* Ring Trial VENoMYC (3).

A differentiation into 55 profiles was achieved, with a discriminatory power (D) of 0.97. The four most discriminatory loci in decreasing order were VNTR3232 (0.83), ETRA (0.64), ETRB (0.54) and QUB26 (0.34).

This set of loci resulted highly discriminatory for the Spanish *M. bovis* SB0121 spoligotype and therefore indicates limited discriminatory power of DVR- spoligotyping in this particular case. The use of an extra panel of loci to discriminate this spoligotype would not contribute additional information.

References:

1. Allix, C., Walravens, K., Saegerman, C., Godfroid, J., Supply, P., Fauville-Dufaux, M. 2006. Evaluation of the Epidemiological Relevance of Variable-Number Tandem-Repeat Genotyping of *Mycobacterium bovis* and Comparison of the Method with IS*6110*

Restriction Fragment Length Polymorphism Analysis and Spoligotyping. J Clin. Microbiol. 44, 1951-1962.

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Advances in Workpackage 6: Molecular characterisation of *M. bovis* and *M. caprae* isolates focused on epidemiological investigation

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Workpackage 6 of FP7-KBBE-2007-212414 (TB-Step) comprises two tasks, namely Task 6.1. To determine the relative contribution of each factor to the maintenance of *M. bovis* transmission, and Task 6.2. To understand the role of *M. caprae* in domestic animals and wildlife.

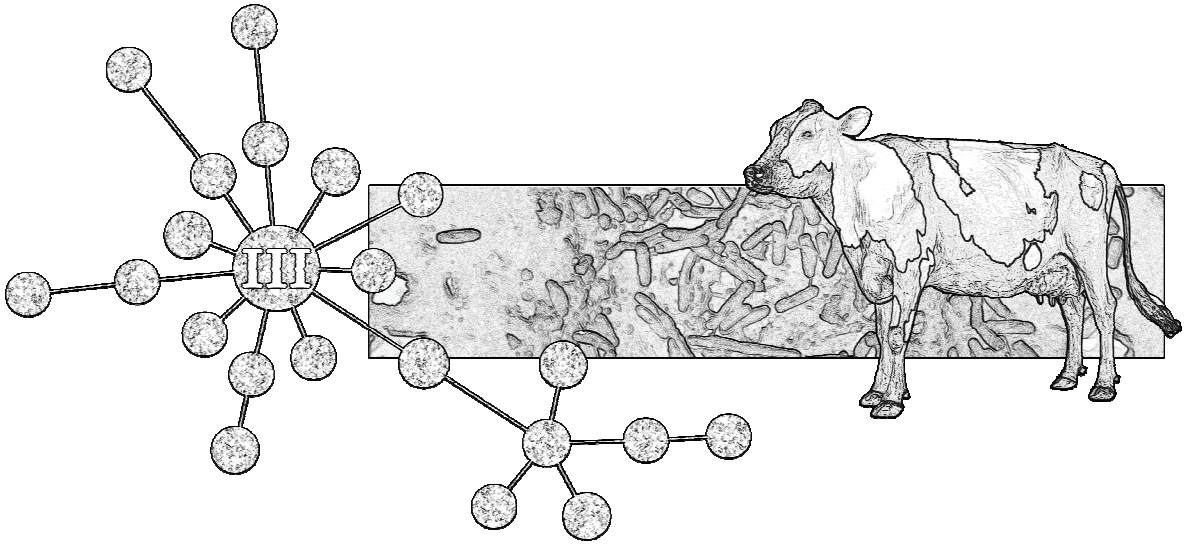
The publications of two surveys of the Spanish *M. bovis* and *M. caprae* population are resumed. The study of *M. bovis* comprised the spoligotyping results of 6215 *M. bovis* isolates that cluster in 252 different spoligotypes and were obtained from 13 animal species, though most frequently (89%) from cattle. The most frequent spoligotype in Spain was SB0121 and approximately 67% of the isolates lacked spacer 21 in their spoligotype pattern. The ongoing studies in collaboration with the Veterinary Laboratories Agency (Weybridge, UK) are commented. The study conducted in 791 *M. caprae* isolates identified 15 spoligotypes which were additionally confirmed as *M. caprae* by RD4 deletion PCR. The samples were collected from seven animal species, and interestingly a significant increase of bovine tuberculosis due to *M. caprae* has been observed in recent years.

The first case of alpaca tuberculosis in Spain and the attempt to trace back this outbreak by MIRU-VNTR typing are described. The study included 30 isolates from cattle within a radius of 150 km around the two related alpaca farms. No matching profile could be identified. The possibility of wildlife acting as a link between cattle and alpacas and the fast evolution of MIRU-VNTR loci, especially QUB3232 and QUB11a are discussed. The centralisation of molecular typing data is emphasized. Moreover, the selection of MIRU-VNTR markers for Spanish isolates is described.

The collaboration with Dr. Szilard Janosi from the Hungarian national reference laboratory (Partner 9), which is focused on the MIRU-VNTR typing of 160 *M. caprae* isolates of Eastern European origin, is commented.

Chapter III

The Spanish national database of animal tuberculosis - mycoDB.es



The Spanish national database of animal tuberculosis - mycoDB.es

The most widely used molecular typing methods for *M. bovis* and *M. caprae* are nowadays spoligotyping (Kamerbeek *et al.*, 1997) and MIRU-VNTR analysis (Frothingham and Meeker-O'Connell, 1998; Supply *et al.*, 2000). Large-scale typing generates vast amounts of data that contribute to a more refined understanding of the epidemiology of animal tuberculosis but also involves the problem of storing the data in a way that makes them easily accessible and analysable.

As part of the current Spanish eradication programme of bovine tuberculosis a database has been developed together with the Ministry of the Environment, and Rural and Marine Affairs (MARM) in order to centralise molecular typing data from all over the country (www.mycoDB.es). Access to the webpage is restricted to Official Veterinary Services and Regional Laboratories involved in the national bovine tuberculosis eradication programme; however, a version of the database that contains simulated information is publicly available for demonstration purposes (Figure 21).

The database with acronym "mycoDB.es" is provided with a geographic viewer which generates maps that show the distribution of spoligotypes, MIRU-VNTR types or the combination of both with municipalities being the smallest geographical unit shown. Users are furthermore able to access the information resumed in the maps in tables including the following epidemiological information: date of isolation, Autonomous Community, province, municipality, animal species, spoligotype and MIRU-VNTR type when available. At first, the database only included spoligotyping data that are available for isolates sampled between 1996 and present; the number of spoligotyped isolates is rising continuously with every periodical update of the database (15.086 *M.* bovis, 1.221 *M. caprae* and six *M. tuberculosis* isolates, consulted on 19th September 2011). In 2011, mycoDB.es was re-designed and extended to MIRU-VNTR typing data which are available for a selection of 357 *M. bovis* isolates, subtyped into 129 different MIRU-VNTR types, and 21 *M. caprae* isolates, subtyped into 15 MIRU-VNTR types (consulted on 19th September 2011). The database offers four possible queries: Spoligotype Search, MIRU-VNTRtype Search, Isolate Search and Isolate maps. Apart from the query tools, brief descriptions of the two molecular typing techniques and related national publications are provided; moreover, a user manual of the main functionalities can be downloaded.

The creation of this national database has been made possible by the collaboration of the National Central Laboratory of Animal Health, the Regional and Provincial Laboratories, Research Institutions and Official Veterinary Services and reflects the commitment at national level to the eradication of animal tuberculosis.

Figure 21. Welcome page of the Spanish database of animal tuberculosis mycoDB.es.

Supplemental material to the following article is available at http://www.sciencedirect.com/science/article/pii/S1567134811003649. Supplementary Table 1 contains the 401 spoligotype patterns (nomenclature according to www.mbovis.org), 379 of which correspond to *M. bovis* strains, 17 to *M. caprae* and five to *M. tuberculosis*. Supplementary Table 2 lists the 172 MIRU-VNTR types obtained with nine loci in the following order: VNTR2165 (ETR-A), VNTR2461 (ETR-B), VNTR580 (ETR-D, MIRU4), VNTR3192 (ETR-E, MIRU31), VNTR2996 (MIRU26), VNTR2163a (QUB11a), VNTR2163b (QUB11b), VNTR4052 (QUB26) and VNTR3232 (QUB3232).

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III.1 A national database of animal tuberculosis - mycoDB.es

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A database for animal tuberculosis (mycoDB.es) within the context of the Spanish national programme for eradication of bovine tuberculosis

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ABSTRACT

Spoligotyping and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) analysis are the international standard techniques for molecular typing of members of the *Mycobacterium tuberculosis* complex. To enable the exploitation of molecular typing data for epidemiological purposes, the creation of large databases is indispensable. Here we describe mycoDB.es, a database for animal tuberculosis which forms part of the Spanish national programme for eradication of bovine tuberculosis. This database has been created as an epidemiological tool at national level and contains spoligotype patterns of 17,273 isolates clustered in 401 different spoligotypes of *Mycobacterium bovis*, *Mycobacterium caprae* and *M. tuberculosis*. The database offers an overview of the present spoligotypes, to a lower extent also of MIRU-VNTR types, affected animal species and furthermore of the spatial distribution of these genotypes.

1. Introduction

The *Mycobacterium tuberculosis* complex (MTBC) includes the main aetiological agents of bovine and caprine tuberculosis, *Mycobacterium bovis* and *Mycobacterium caprae*. Both pathogens can cause disease in a wide range of animal species and also in humans. Since implementation of molecular fingerprinting of MTBC isolates (van Embden et al., 1993), several techniques have contrib-

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uted to a more refined understanding of infectious disease epidemiology (Durr et al., 2000; Moonan et al., 2009). The most widely used molecular typing methods are nowadays spoligotyping (Kamerbeek et al., 1997) and MIRU-VNTR analysis (Frothingham and Meeker-O'Connell, 1998; Supply et al., 2000).

The first database for molecular typing results, created by the Institute Pasteur Guadeloupe focused on human tuberculosis and included information on spoligotyping results of a limited number of *M. tuberculosis* isolates from few geographical settings (Sola et al., 1999). By systematically collecting published spoligotypes a better representation of the worldwide *M. tuberculosis* diversity was achieved (Sola et al., 2001; Filliol et al., 2003). The latest update of this database, SpolDB4 (Brudey et al., 2006), provides a higher resolution image of the worldwide *M. tuberculosis* genome diversity and enables large-scale studies of epidemiology and population genetics, including spoligotyping and MIRU-VNTR typing results, in total 39,609 entries from 121 countries (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html, consulted on 10th April 2011). MIRU-VNTRplus (http://www.miru-vntrplus.org/MIRU/index.faces) is a new database created with a similar purpose (Allix-Béguec et al., 2008; Weniger et al., 2010). Apart from the spoligotyping pattern and a 24-locus MIRU-VNTR profile it additionally includes information on single-nucleotide- and large-sequence-polymorphisms leading to optimal phylogenetic identification. Both databases are freely accessible, but unfortunately their use for bovine TB epidemiology is limited due to a low number of animal isolates. In response to the need of an international nomenclature for spoligotypes of animal origin, in 2003 the *M. bovis* spoligotype database was created (Smith and Upton, in press, this issue). The implementation of a common nomenclature has provided an easy way to compare spoligotyping results and hence is of great value for the scientific community.

Herein we describe a national database for spoligotyping and, to a lower extent, MIRU-VNTR typing data of animal tuberculosis which has been created as a tool for molecular epidemiology within the Spanish national eradication programme for bovine tuberculosis.

2. Material and methods

2.1. Informatics

The mycoDB database is accessible via a XHTML/CSS web application implemented in php language running in an Apache Web Server (The Apache Software Foundation, MD) under Linux (The Linux Kernel Organization Inc., CA). The mycoDB database makes use of AJAX technologies (Asynchronous JavaScript and XML, OpenAjax Alliance) and JavaScript must be activated in the browser.

On the main page, a brief description of the techniques is provided and the user manual of the main functionalities can be downloaded. Four possible queries are available: spoligotype search, MIRU-VNTR type search, isolate search and isolate maps. Each search summarizes the results in a box and displays the retrieved data in a map showing their geographical distribution. Moreover, the detailed data are shown in a series of tables, namely isolate table, MIRU-VNTR table, region table, animal table, spoligo table and data table. The query result pages use ALOV Map Java Applet (TimeMap Project, University of Sydney) (Johnson, 2004), a web-based geographic viewer where maps are described by xml files, and Java software (Oracle Corporation, CA) is necessary for correct display. The smallest geographical unit considered in the database and therefore shown in the maps is the municipality (basic local entity of the Spanish government). This geographical information system (GIS) allows the visualisation of data in a map with variable size detail.

Access to the webpage is restricted to Official Veterinary Services and Regional Laboratories involved in the national bovine tuberculosis eradication programme. For demonstration purposes a demo version of mycoDB which contains simulated information is available at http://www.mycodb.es; any resemblance with reality is purely coincidental.

2.2. Data collection

The 17,273 isolates included in the database were collected from 1996 until 2011 in the national territory. The control of bovine tuberculosis is defined by the Spanish Programme on Eradication on Bovine Tuberculosis which stipulates the inclusion of spoligotyping results in the national database since 2008. The Regional Laboratories collaborate at different stages in the tuberculosis analysis, so that at the Centro de Vigilancia Sanitaria Veterinaria (VISAVET) different types of samples can be received: tissue samples for culture (to date 37% of the samples), mycobacterial cultures (15%), DNA (47%) or spoligotyping data (1%). Monthly updates are performed to actualise mycoDB with the latest results.

At present, data include *M. bovis* ($n = 15{,}898$), *M. caprae* ($n = 1369$) and *M. tuberculosis* ($n = 6$) isolates from cattle (*Bos taurus*, $n = 14{,}723$), domestic goats (*Capra aegagrus hircus*, $n = 1078$), domestic pigs (*Sus scrofa domestica*, $n = 179$), sheep (*Ovis aries*, $n = 15$), alpacas (*Lama pacos*, $n = 3$), wild boar (*Sus scrofa*, $n = 618$), red deer (*Cervus elaphus*, $n = 282$), fallow deer (*Dama dama*, $n = 348$), Iberian lynxes (*Lynx pardinus*, $n = 5$), foxes (*Vulpes vulpes*, $n = 4$), chamois (*Rupicapra rupicapra*, $n = 2$), badgers (*Meles meles*, $n = 9$), cats (*Felis silvestris catus*, $n = 3$), a dog (*Canis lupus familiaris*, $n = 1$), a yellow-crowned parrot (*Amazona ochrocephala*, $n = 1$), a mouflon (*Ovis musimon*, $n = 1$, zoo animal), and a peccary (*Tayassusu* sp. $n = 1$, zoo animal).

2.3. Molecular typing

Spoligotyping is performed according to Kamerbeek et al. (1997). Assignation of *M. tuberculosis* complex organisms to *M. bovis*, *M. caprae* or *M. tuberculosis* species is based on recognition of specific spoligotyping signatures plus additional identification of absence/presence of region of difference (RD)4 and RD9 (Mostowy et al., 2002; Brosch et al., 2002) for confirmation of the preassigned types.

MIRU-VNTR typing is carried out as per Frothingham et al. (1998) and Supply (2006). The loci used for MIRU-VNTR typing are the six loci recommended by VENoMYC (Supply, 2006) [VNTR2165 (ETR-A), VNTR2461 (ETR-B), VNTR580 (ETR-D, MIRU4), VNTR2163a (QUB11a), VNTR2163b (QUB11b) and VNTR3232 (QUB3232)] plus additional three loci [VNTR3192 (ETR-E, MIRU31), VNTR2996 (MIRU26), VNTR4052 (QUB26)] (Supply et al., 2001; Skuce et al., 2002).

3. Results

3.1. Database organization

The database, mycoDB, is operating since 2007 and is available in Spanish and English. Its use is intuitive, providing necessary information on spoligotyping and MIRU-VNTR analysis on the welcome page. A large amount of results included in this database has been previously published and information of these publications is at hand on the publicly accessible demo version. The database allows users to recall data through four different types of request, namely spoligotype search, MIRU-VNTR type search, isolate search and isolate maps.

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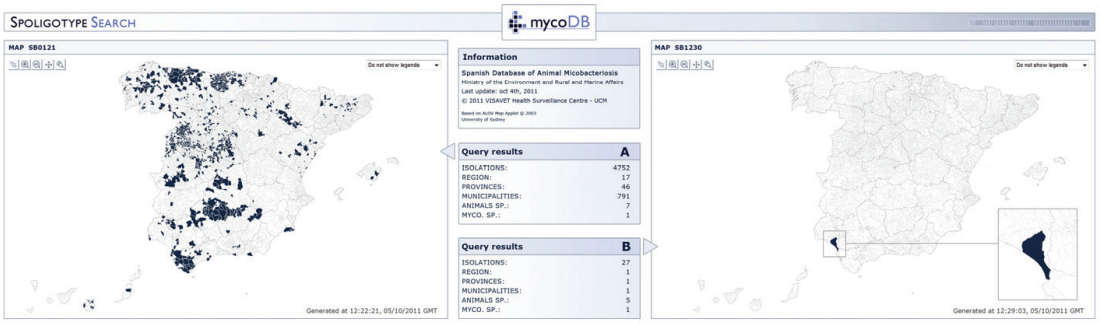

Fig. 1. Maps obtained with spoligotype search showing differential distribution of spoligotypes in Spain. Map of the most frequent spoligotype SB0121 in comparison with a map of a geographically localised profile, SB1230, in Doñana National Park.

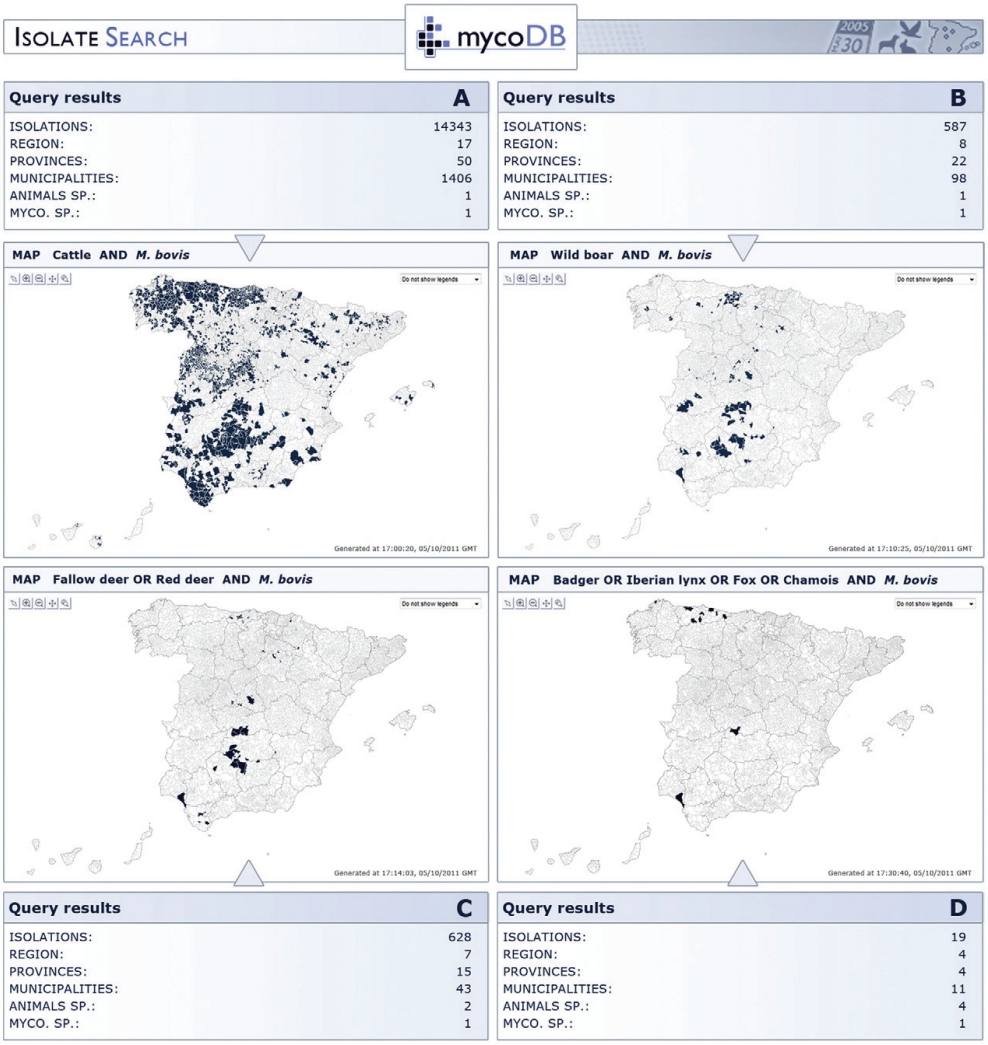

Fig. 2. Maps of the presence of *Mycobacterium bovis* in cattle and in different wildlife species in Spain. These have been obtained performing the isolate search selecting the different animal species. (A) Cattle; (B) Wild boar; (C) Red deer/fallow deer; (D) other species (badger, Iberian lynx, fox and chamois).

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3.2. Spoligotype search

The spoligotype search offers the possibility to explore a distinct spoligotype pattern using the international nomenclature (SB or SIT number) or the internal nomenclature for spoligotypes. The search also offers a conversion tool from the binary code of a spoligotype pattern to the official or internal name and vice versa. This query provides an overview of the geographical distribution of a spoligotype and of the animal species from which it has been isolated. To date, the isolates cluster in 401 different spoligotype patterns, 379 of which correspond to *M. bovis* strains, 17 to *M. caprae* and five to *M. tuberculosis* (Supplementary Table 1). The highest spoligotype diversity of *M. bovis* was found in cattle (350 spoligotype patterns out of 13166 isolates) followed by wild boar (43 types out of 450 isolates). As an example Fig. 1 shows maps of the distribution of the most frequent spoligotype in Spain, SB0121 (Rodríguez et al., 2010), and a geographically localised profile, SB1230, in Doñana National Park (Romero et al., 2008).

3.3. MIRU-VNTR type search

MIRU-VNTR type search retrieves every isolate with the requested type [entered either as number of repeats for each locus or as composite (numbers separated by hyphens)]. The MIRU-VNTR type can include up to nine loci, if the result for a locus is unknown it appears as "?" and if the amplification failed as "F". Information includes the *M. tuberculosis* complex organism, the spoligotype, plus same information as above. Currently, 389 *M. bovis* isolates were subtyped into 157 different MIRU-VNTR types and 21 *M. caprae* in 15 MIRU-VNTR types (Supplementary Table 2).

3.4. Isolate search

Isolate search allows for the localisation of isolates combining the above mentioned queries of typing patterns with independent or combined selection criteria such as year of isolation (from 1996 to 2011) or periods of time, geographical spread [Autonomous Community ($n = 17$), province ($n = 50$), municipality ($n = 8810$)], and animal species ($n = 18$). As before, the information is shown in a table and displayed in a map. This query is useful for epidemiological studies since it enables the visualisation of transmission between different regions or between wildlife and livestock; Fig. 2 shows the presence of *M. bovis* in different wildlife species in Spain.

This tool also performs queries regarding a mycobacterial species. At the moment, 1369 *M. caprae* isolates are registered, 27.3% of which occurring in cattle and not necessarily linked to the presence of goats (Fig. 3), a fact that underlines the observation of an increase of *M. caprae* in cattle in Spain (Rodríguez et al., 2011).

3.5. Isolate maps

Additionally, annual isolate maps that display the respective positive isolations centralised in the database are at the user's disposal. These isolate maps can be used as a control for regional participation. Fig. 4 shows the isolate map for the year 2010 in comparison with the corresponding annual herd prevalence map for cattle.

4. Discussion

In this report we describe the development and the operation of a database of genotyping of pathogens causing animal tuberculosis. *Mycobacterium bovis*, and to a lesser extent *M. caprae*, are successful animal pathogens in Spain; extensive human and economic efforts directed toward eradication achieved reduction of the herd prevalence from 2.11% to 1.51% in the last decade, costing over 50 million € per year (unpublished data, MARM 2010).

This database has resulted from the commitment of numerous contributors to the study of the epidemiology of animal tuberculosis. This is reflected on the annual isolate maps, which emphasises the progress in using and centralising molecular data made since 1996 and the almost complete coverage of the national territory. Understanding the epidemiology of the infection should be a collaborative task that benefits from the science-policy interface (Task Force Bovine Tuberculosis Subgroup, 2006; Proceedings of the 4th International Conference on *Mycobacterium bovis*, 2006). It requires the collection of a large amount of information combining classical and molecular data. Although a high number of entries difficult the management of data, the design of mycoDB intends to be intuitive and user-friendly and offers the possibility of both large-scale and local enquiries. The exploitation of the database provides support to the control programme. On the one hand, results have been applied to increase our knowledge of the demography of both pathogens in a broad context (Rodríguez et al., 2010, 2011) and to corroborate transmission of *M. bovis* between wildlife and domestic animals (Romero et al., 2008). On the other hand, it would be useful to explain the relevance of small-scale transmission at local or farm level, such as relative contribution of reactivation of latent infection in the farm or exogenous infection through contact by trade or communal pastures. On long term basis, this could offer clues about the existence of isolates of atypical behaviour, for example emerging strains with increased clonal

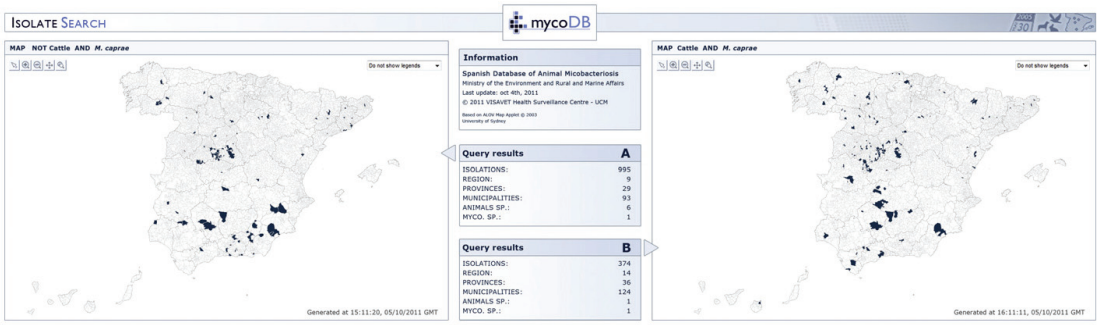

Fig. 3. Distribution of *Mycobacterium caprae* in cattle and other species using the isolate search tool by combining mycobacterial and animal species criteria. The search retrieves 1369 isolates affecting seven animal species in a total of 197 municipalities.

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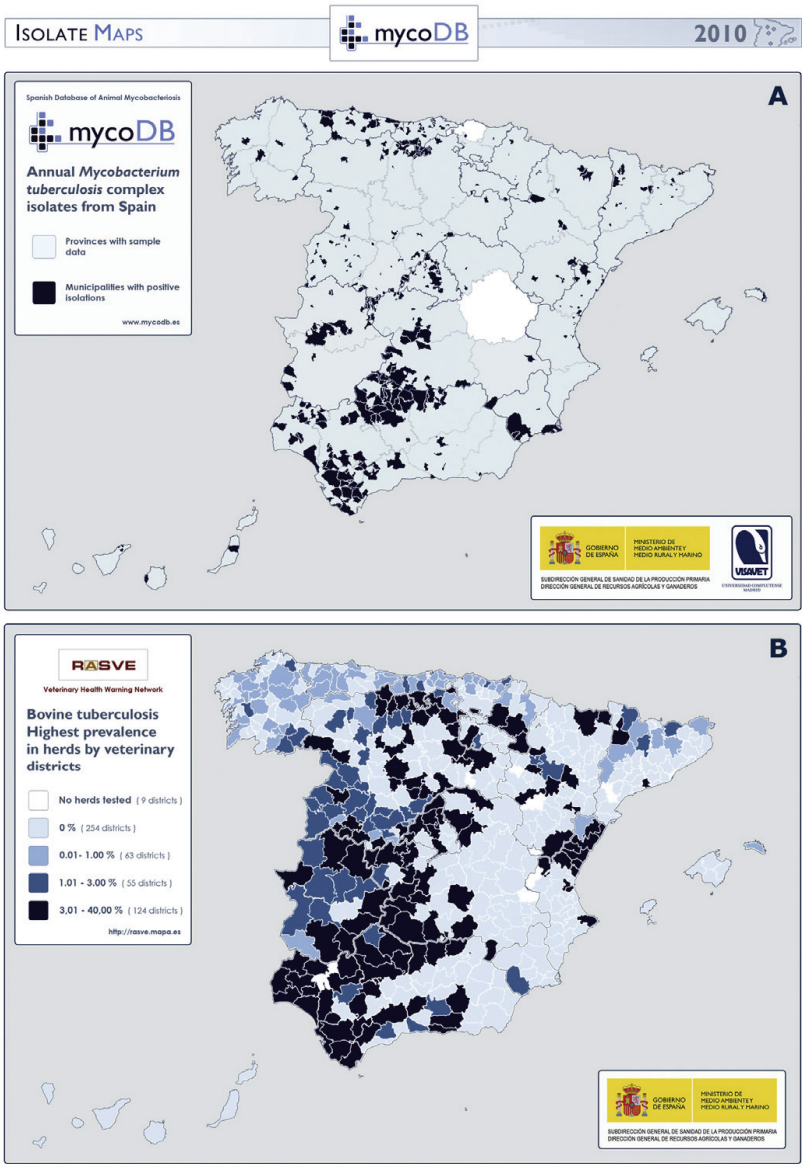

Fig. 4. Comparison of the isolate map for the year 2010 shows that the collection of molecular typing information is consistent with the corresponding annual herd prevalence map for cattle.

expansion, hypervariable isolates or isolates which may induce altered immune response that hinder diagnosis.

Although the database has been successful in many ways, we are aware that there are several possibilities for improvement. The search based on the spoligotype search tool may in some situations present limitations, because the analysis is currently on perfect match basis. This could be misleading, because epidemiologically related, but not-matching isolates, for example due to deletion of a DVR or block of DVRs or convergence due to homoplasy are not considered; in these cases, the combined interpretation of profiles along with classical epidemiological information and the focused additional use of MIRU-VNTR analysis are required to unravel the potential link (Romero et al., 2006). However, the search for a certain MIRU-VNTR type has only recently been implemented and therefore contains less data. This search for spoligotype patterns could be modified to a less stringent criterion, in order to allow for a degree of mutation in the profile to be compared.

Members of the *M. tuberculosis* complex are also serious human pathogens. The mycoDB database can improve data exchange between public health and veterinary services; therefore, it can help to fully recognise the role as a zoonosis and therefore the value of the eradication programme in terms of human health. Since 1989

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the microbiological information system regarding cases of *M. bovis* and *M. caprae* affecting humans in Spain had been based on voluntary weekly reporting by the laboratories; currently, notification has become compulsory. Comparison of patterns can include as well the primary human pathogen *M. tuberculosis*. Isolations of *M. tuberculosis* from samples of animal origin are rare findings (Prasad et al., 2005; Jenkins et al., 2011) though the first cases in Spain have recently been described (Romero et al., accepted).

Providing the GIS of the application with orthophotographs and parcel of land layers is one of the future objectives which will improve the visualisation of overlapping livestock and wildlife habitats and shared pastures.

In conclusion, the national database is being used as a valuable tool for animal tuberculosis epidemiology and forms one of the pillars of the current Spanish national eradication programme. We strongly encourage the use of databases for centralisation of molecular typing results and emphasise the importance of a comprehensive collaboration between the veterinary and public health services.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.10.008.

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III.2 Contributions to conferences and meetings of European projects

Rodríguez S. The national database of *Mycobacterium bovis* and *Mycobacterium caprae*. Use in epidemiological surveys. Oral presentation. Meeting of the bovine tuberculosis subgroup of the Task Force (EFSA). Seville (Spain), 14-15 November 2007.

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de Juan L., **Rodríguez S.**, Romero B., Aranaz A., Bezos J., Castellanos E., González S., Sáez J. L., Mateos A. and Domínguez L.. Spanish database of animal mycobacteriosis (mycoDB): application in epidemiological studies. Oral presentation. 31st Annual Congress of the European Society of Mycobacteriology. Bled (Slovenia), 4-7 June 2010.

The national database of *Mycobacterium bovis* and *Mycobacterium caprae*. Use in epidemiological surveys.

Rodríguez S.

Laboratory VISAVET, Departamento Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain.

[The national database on *M. bovis* and *M. caprae* isolates was presented. This database contains 36,900 samples from 1996-2007, including 264 distinct spoligotypes of the two species *M. bovis* and *M. caprae*. The database will be extended to include more data as well as a GIS function so that spoligotypes can be displayed on country maps. Currently, information about the location, identity and species of the sampled animal, as well as the bacterial species, spoligotype and date of receipt of the sample is stored.] REPORT OF THE "BOVINE TUBERCULOSIS" SUB-GROUP TASK FORCE, SANCO/10584/2007.

http://ec.europa.eu/food/animal/diseases/eradication/reportsanco-10584-2007btbsubgroupsevillarev110-1-08.pdf

The national database on Spanish *Mycobacterium bovis* and *Mycobacterium caprae* isolates.

Rodríguez S., Romero B., de Juan L., González S., Bezos J., Álvarez J., Castellanos E., Lozano F.J., Moya N., Álvarez N., Alende T., Gutiérrez A., Gallardo F., Mateos A., Aranaz A. and Domínguez L.

Centre VISAVET, Universidad Complutense de Madrid, Madrid, Spain.

Due to the importance of molecular typing for epidemiological studies, the Animal Health Surveillance Centre VISAVET created a national database on *Mycobacterium* (*M.*) *bovis* and *M. caprae* isolates in a collaboration with the Spanish Ministry of Environmental, Rural and Marine Affairs (MARM, former Ministry of Agriculture, Fisheries and Food).

The database centralizes the spoligotyping results of Spanish *M. bovis* and *M. caprae* isolates. Currently, the database contains the spoligotyping results of 9394 isolates collected since 1996. The 8698 *M. bovis* isolates present 240 different spoligotypes, and the 696 *M. caprae* isolates 11 different spoligotypes.

Information about the origin and species of the sampled animal, as well as the date of receipt of the sample is stored. Data are represented in tables and maps. The maps will be complemented by a Geographical Information System (GIS). The first steps are taken to include the national data of MIRU-VNTR typing as well.

The database is considered a useful tool to reinforce the national eradication programme and is accessible on the homepage of the Veterinary Health Alert Network (RASVE - MARM). It can be consulted by the National and Regional Authorities which are implicated in the eradication of bovine tuberculosis (bTB).

Spanish database of animal mycobacteriosis

Romero B.[1, 2], **Rodríguez S.**[1, 2], Bezos J.[1, 2], Álvarez J.[1], Castellanos E.[1, 2], González S.[1], Lozano F.[1], Moya N.[1], Gutiérrez A.[1], Alende T.[1], Sáez J. L.[3], Mateos A.1, 2, Aranaz A.[1, 2] and Domínguez L.[1, 2]

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[3] Spanish Ministry of the Environment and Rural and Marine Affairs.

VISAVET Health Surveillance Centre, in collaboration with the Spanish Ministry of the Environment and Rural and Marine Affairs (MARM), is the institution responsible for the database where national data regarding animal mycobacteriosis are registered since 1996 to the present day. This database has an additional viewer of mycobacterial isolations according to searching criteria introduced by the user, for instance the year of isolation, host species from which the bacteria was cultured, geographical origin of the sample or spoligotypes involved.

Currently, the database contains the spoligotyping results of 11.090 isolates collected since 1996. The M. bovis isolates (n=10.341) are grouped in 308 spoligotypes, and the M. caprae isolates (n=749) in 11 different spoligotypes. Information about the origin and species of the sampled animal, as well as the date of receipt of the sample is stored. Data are represented in tables and maps. The maps will be complemented by a Geographical Information System (GIS). Moreover, the first steps are taken to include the national data of MIRU-VNTR typing.

The database is considered a useful tool to reinforce the national eradication programme and is accessible on the homepage of the Veterinary Health Alert Network (RASVE - MARM). It can be consulted by the National and Regional Authorities which are implicated in the eradication of bovine tuberculosis.

VISAVET HEALTH SURVEILLANCE CENTRE
COMPLUTENSE UNIVERSITY OF MADRID

SPANISH DATABASE OF ANIMAL MYCOBACTERIOSIS

Beatriz Romero[1, 2], Sabrina Rodríguez[1, 2], Javier Bezos[1, 2], Julio Álvarez[1], Elena Castellanos[1, 2], Sergio González[1], Francisco Lozano[1], Nuria Moya[1], Alexandra Gutiérrez[1], Tatiana Alende[1], José Luís Sáez[3], Ana Mateos[1, 2], Alicia Aranaz[1, 2], Lucas Domínguez[1, 2]

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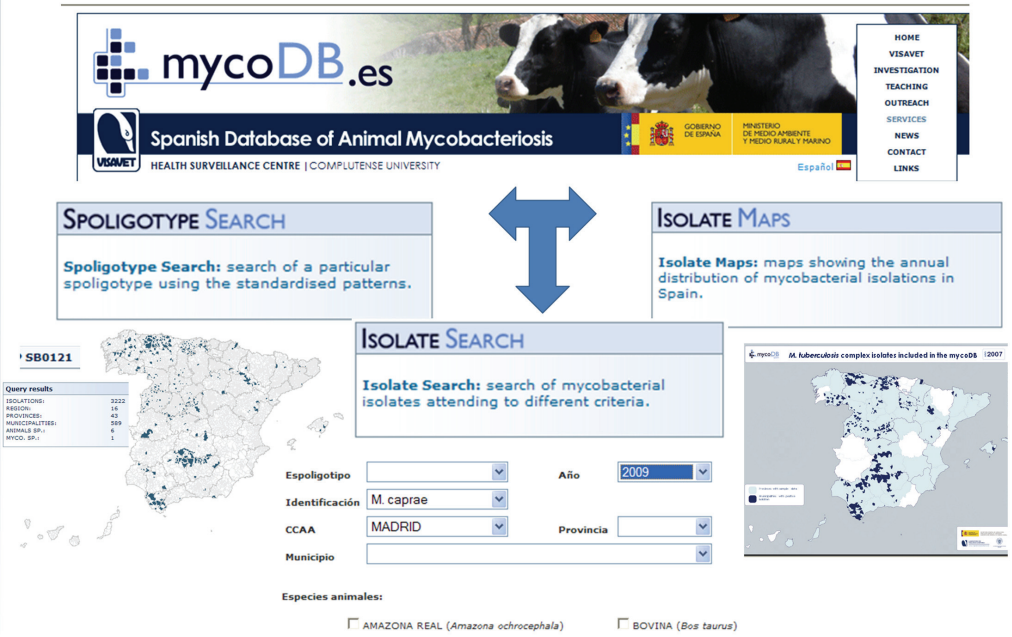
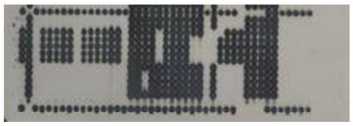
VISAVET Health Surveillance Centre, in collaboration with the Spanish Ministry of the Environment and Rural and Marine Affairs (MARM), is the institution responsible for the database where national data regarding animal mycobacteriosis (mainly caused by *M. bovis*/*M. caprae*) are registered since 1996 to the present days. This database has an additional viewer of the geographic distribution of mycobacterial isolations according to searching criteria introduced by the user.



The database comprises the spoligotyping results of 11.090 isolates divided in 319 spoligotypes. Information about the origin and species of the sampled animal, as well as the date of receipt of the sample is stored. Data are represented in tables and maps. Moreover, the first steps are taken to include the national data of MIRU-VNTR typing.

mycoDB (n=11.090)	Species	Isolates	Spoligotyping profiles
	M. bovis	10.341	308
	M. caprae	749	11



The database is considered a useful tool to reinforce the national eradication programme and is accessible on the homepage of the Veterinary Health Alert Network (RASVE - MARM). It can be consulted by the National and Regional Authorities which are implicated in the eradication of bovine tuberculosis.

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Spanish database of animal mycobacteriosis (mycoDB): Application in epidemiological studies

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Epidemiological studies based on molecular characterization have allowed a better understanding of several factors as for example transmission between domestic and wildlife animals, animal movement, outbreaks, etc. The Direct Variable Repeat Spacer Oligonucleotide Typing technique or DVR-spoligotypiing is based on polymorphism of the chromosomal DR locus, which consists of a variable number of direct repeats (DR) interspersed with nonrepetitive spacers. This technique is specific for bacterial species included in the M. tuberculosis complex and nowadays, this technique is the method of choice for epidemiological studies.

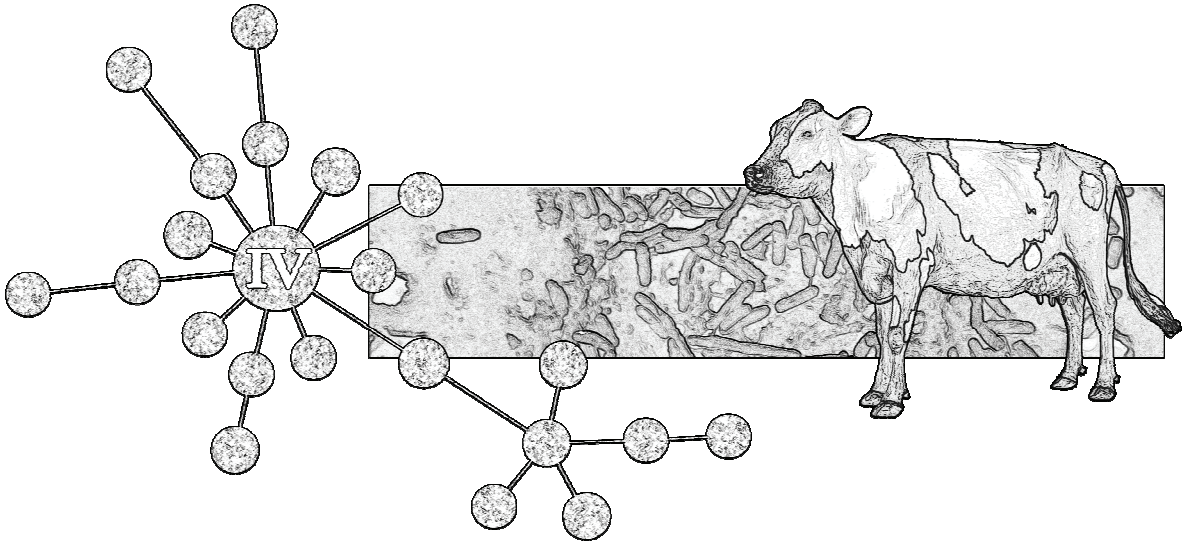
Due to the importance of epidemiological studies in tuberculosis together with the Ministry of the Environment and Rural and Marine Affairs (MARM) we designed a Spanish Database of Animal Mycobacteriosis (mycoDB) which includes the national isolates of *M. bovis* and *M. caprae* since 1996. The accesss is restricted to Veterinary Services and Laboratories involved in the National Eradication Program. The access is available at the Veterinary Health Alert Network website (RASVE). The database offers three different searches by spoligotype, isolate and maps.

Currently, the database contains 13,731 *M. bovis* strains grouped in 319 spoligotypes and 919 *M. caprae* strains classified in 15 spoligotypes. During the presentation examples of the application of the database in epidemiological studies will be shown at three levels: 1) identification of outbreaks; 2) transmission between domestic and wildlife animals; and 3) impact of bovine tuberculosis in Public Health.

The Spanish Database of Animal Mycobacteriosis (mycoDB) is a useful tool for epidemiological studies at a national and international level since a standardized protocol and nomenclature is used. We encourage all countries to centralize all the typing information in a database for surveillance and epidemiological studies purposes.

Chapter IV

Phylogeny of *Mycobacterium bovis* in the Iberian Peninsula



Phylogeny of *Mycobacterium bovis* in the Iberian Peninsula

The population structure of the *Mycobacterium tuberculosis* complex is highly clonal since no signs of transfer and recombination of chromosomal sequences between strains have been identified; in a highly clonal population genetic features such as large sequence polymorphism (LSP) and single nucleotide polymorphism (SNP) are passed on to all the descendants (Smith *et al.*, 2006). The Direct Repeat (DR) region is exploited by spoligotyping for molecular epidemiology purposes, but can furthermore be useful in population genetics due to the unidirectional evolution of this locus occurring by single spacer deletions or loss of contiguous spacer sequences (Fang *et al.*, 1998; van Embden *et al.*, 2000). Distinct characteristics of spoligotype patterns, named spoligotype signatures (Streicher *et al.*, 2007), can be used as indicators of certain lineages of *M. tuberculosis* and *M. bovis* (Kato-Maeda *et al.*, 2011; Smith *et al.*, 2011).

Large surveys of the *M. bovis* populations in Portugal (Duarte *et al.*, 2008) and Spain (Rodríguez *et al.*, 2010) revealed a spoligotype signature characterised by the loss of spacer 21 which is common to 70% of *M. bovis* isolates in the Iberian Peninsula. In order to screen for a possible phylogenetic marker in *M. bovis* strains with spacer 21-deleted spoligotypes DNA microarrays were carried out in four Spanish strains lacking spacer 21, but no large sequence polymorphism was identified that would be a suitable phylogenetic marker. Subsequently, three *M. bovis* strains with spacer 21 missing were subjected to whole genome sequencing and analysed for SNPs common to the three strains but absent from reference strains *M. bovis* AF2122/97, *M. bovis* BCG and *M. tuberculosis* H37Rv; 108 SNPs were identified that matched this criterion. Out of the 108 SNPs we selected 16 that were then screened in ten Spanish *M. bovis* isolates. The SNP at nucleotide position 3765573 (*M. bovis* AF2122/97) of the gene *guaA*, that encodes a probable guanine monophosphate synthetase, is a synonymous nucleotide change in a triplet encoding alanine and was able to distinguish the spacer 21-deleted strains from the strains with spacer 21 present in their spoligotype pattern. In order to circumvent costly sequencing, a PCR-restriction endonuclease analysis (PCR-REA) was set up for the assessment of this SNP in representative sets of *M. bovis* isolates from Spain (n=201), Portugal (n=48), France (n=145) and Italy (n=50). The absence of spacer 21 together with the SNP in *guaA* defines a clonal complex of *M. bovis*, called European 2 (Eu2), dominant in the Iberian Peninsula, present at low frequency in France and Italy, and absent from the British Isles.

The spoligotypes identified in Spain also comprise spoligotypes that present characteristic features that hint at other clonal complexes, such as the African 2 (Af2) and European 1 (Eu1) clonal complex. In order to assess the proportion of these clonal

complexes within the Spanish *M. bovis* population, two sets of selected *M. bovis* isolates were screened for the deletions RDAf2 (n=20) and RDEu1 (n=80).

M. bovis is present in most African countries and to date two different clonal complexes have been described in the African continent, African 1 (Af1) (Müller *et al.*, 2009) and African 2 (Af2) (Berg *et al.*, 2011). In East Africa a spoligotype signature marked by the absence of spacers 3 to 7 has been observed and a 14.1 kb deletion comprising the whole *mce2* operon, called Region of Difference (RD) Af2, was identified in African strains with this feature by DNA microarray; a simple deletion PCR was developed to determine the presence or absence of RDAf2. The combination of absence of spacers 3 to 7 from the spoligotype pattern and deletion of RDAf2 defines a clonal complex, named Af2, which is prevalent in Uganda, Burundi, Tanzania and Ethiopia. In Spain less than 1% of *M. bovis* isolates show the spoligotype signature of Af2 strains and typing of 20 isolates matching the Af2 signatures that are included in the paper "African 2, a clonal complex of *Mycobacterium bovis* epidemiologically important in East Africa" did not reveal the RDAf2 deletion, leading to the conclusion that the Af2 clonal complex is absent from Spain (Berg *et al.*, 2011).

In the United Kingdom and the Republic of Ireland most *M. bovis* isolates lack spacer 11 in their spoligotype pattern and a deletion, RDEu1, was identified that is closely related with this spoligotype signature. The RDEu1 deletion, formerly RD17 (Gordon *et al.*, 2001), is 806 bp long and located within the *treY* gene which encodes the malto-oligosyltrehalose synthase, an important enzyme in the biosynthesis of the disaccharide trehalose (De Smet *et al.*, 2000). The RDEu1 deletion together with the loss of spacer 11 in the spoligotype pattern defines a clonal complex of *M. bovis* that is at virtual fixation in the British Isles, the Eu1 clonal complex. *M. bovis* isolates with spacer 11 deleted spoligotypes can be found all over the globe and an extensive study was conducted to evaluate the RDEu1 locus in these strains using simple deletion PCR. A total of 80 Spanish *M. bovis* isolates was deletion typed for RDEu1; out of the 80 isolates 21 had spacer 11 and RDEu1 deleted, while 14 isolates had spacer 11 deleted from their spoligotype patterns but were intact at RDEu1. The other 45 isolates had spoligotypes with spacer 11 present and were intact at RDEu1. A representative subset of 45 strains was included in the paper "European 1: a globally important clonal complex of *Mycobacterium bovis*" and the proportion of Spanish strains belonging to the Eu1 clonal complex was estimated 6.1% (Smith *et al.*, 2011).

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IV.1. The European 2 clonal complex of *M. bovis*

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European 2 – A clonal complex of *Mycobacterium bovis* dominant in the Iberian Peninsula

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ABSTRACT

Mycobacterium bovis isolates from the Iberian Peninsula are dominated by strains with spoligotype patterns deleted for spacer 21. Whole-genome sequencing of three Spanish strains with spacer 21 missing in their spoligotype pattern revealed a series of SNPs and subsequent screening of a selection of these SNPs identified one in gene *guaA* that is specific to these strains. This group of strains from the Iberian Peninsula missing spoligotype spacer 21 represents a new clonal complex of *M. bovis*, defined by the SNP profile with a distinct spoligotype signature. We have named this clonal complex European 2 (Eu2) and found that it was present at low frequency in both France and Italy and absent from the British Isles.

1. Introduction

Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex (MTBC), is the main causative agent of bovine tuberculosis, but is able to infect a variety of host species including livestock, wildlife and humans. The MTBC is a highly clonal group of strains (Smith et al., 2006) and genetic diversity among its members may in part be caused by deletions that are expected to represent genetic events (Groenen et al., 1993; Behr and Small, 1999; Gordon et al., 1999). Such deletions, or large sequence polymorphisms, have been exploited in order to create an evolutionary scenario for the MTBC based on regions of difference (RD) (Brosch et al., 2002; Mostowy et al., 2002). Single nucleotide polymorphisms (SNPs) have also been used to establish phylogenies (Gutierrez et al., 2005; Filliol et al., 2006; Smith et al., 2006; Hershberg et al., 2008; Garcia-Pelayo et al., 2009; Schürch et al., 2011).

Direct Variable Repeat Spacer Oligonucleotide Typing (DVR-spoligotyping) (Kamerbeek et al., 1997) is the most widely used molecular typing technique for *M. bovis*. It detects polymorphisms in the Direct Repeat (DR) locus which consists of multiple DR

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sequences interspersed with non-repetitive spacer sequences. Strains vary in the presence and absence of these spacers and thus can be assigned distinct spoligotypes. In the past, descriptions of *M. bovis* populations were often based on the comparison of spoligotypes and certain spoligotype features were found in geographically localised groups of strains. Three new RDs have recently been associated to different spoligotype signatures and have been used to describe two clonal complexes in Africa, African 1 (Af1) (Müller et al., 2009) and African 2 (Af2) (Berg et al., 2011), as well as the globally important clonal complex European 1 (Eu1) which is at virtual fixation in the British Isles (Smith et al., in press; Smith, in press, this issue).

Large population surveys from Portugal (Duarte et al., 2008) and Spain (Rodriguez et al., 2010) revealed that the majority of the *M. bovis* strains (70%) in the Iberian Peninsula lack spacer 21 in their spoligotyping profile, unlike strains from France and Italy whose *M. bovis* populations are dominated by SB0120 (BCG-like) (Haddad et al., 2001; Boniotti et al., 2009). We used DNA microarray analysis to screen Spanish *M. bovis* isolates marked by the absence of spacer 21 for a common deletion, but no phylogenetically informative deletion could be identified. However, whole-genome sequencing detected a SNP specific for spacer 21-deleted spoligotypes which could be a possible marker for this group of strains. We used this SNP to describe a new clonal complex in the Iberian Peninsula which we have named European 2 (Eu2).

2. Material and methods

2.1. Mycobacterial strains

The study included a total of 444 strains originating from Spain ($n = 201$) [strain collection at the Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense de Madrid], Portugal ($n = 48$) [strain collection at the Laboratório Nacional de Investigação Veterinária (LNIV), Lisboa], France ($n = 145$) [strain collection at the Unité Zoonoses Bactériennes, Laboratoire de Santé Animale de Maisons-Alfort, ANSES, Maisons-Alfort Cedex] and Italy ($n = 50$) [strain collection at the Centro Nazionale di Referenza per la Tubercolosi Bovina, IZSLER, Brescia] (Supplementary Table 1). All isolates were obtained from cattle except 18 Spanish strains with common spoligotype patterns that were isolated from a goat ($n = 1$), fallow deer ($n = 2$), red deer ($n = 5$) and wild boar ($n = 10$), and 10 Portuguese strains isolated from red deer ($n = 6$) and wild boar ($n = 4$).

A subset of four spacer 21-deleted Spanish strains was used for DNA microarray analysis; a cattle isolate with spoligotype SB0121 plus three isolates additionally lacking either spacer 6 (SB0265), spacer 37 (SB0295), or spacers 28–30 (SB1337). Three spacer 21-deleted Spanish strains were selected for whole genome sequencing: SB0121, SB0265 and SB0295 (Supplementary Table 1). These isolates were grown in Middlebrook 7H9 broth and whole genomic DNA was obtained following the CTAB method similar to the previously described by van Soolingen et al. (1994). *M. tuberculosis* H37Rv (genomic DNA provided by TB Research Materials and Vaccine Testing Contract at Colorado State University, USA), *M. bovis* AF2122/97 [strain collection at the Animal Health and Veterinary Laboratories Agency (AHVLA), Addlestone, UK], were used as control strains. For comparative molecular typing, *M. bovis* CHAD491 (Af1, Müller et al., 2009) and *M. bovis* BTB1087 (Af2; Berg et al., 2011) were used as representative control strains of the *M. bovis* Af1 and Af2 clonal complexes, respectively.

2.2. Spoligotyping and DNA microarray analysis

Spoligotyping was performed as described by Kamerbeek et al. (1997) in the respective laboratories. Authoritative names (prefix SB followed by four digits) for spoligotype patterns were obtained from the *M.* bovis Spoligotype Database website (http://www.Mbovis.org) (Smith and Upton, in press, this issue).

For microarray analysis 2 μg of whole genomic DNA for each isolate [MI06/01507 (SB0121), MI06/00012 (SB0265), MI05/02665-1 (SB0295) and MI05/02637 (SB1337)] was purified and deletions identified by microarray hybridisation using previously published methods (Garcia-Pelayo et al., 2004). Each isolate was analysed twice. The *M. bovis*/*M. tuberculosis* composite microarrays used in these experiments consisted of 4410 PCR products (size range 60–1000 bp) that represent all the genes in the genomes of *M. bovis* strain AF2122/97 and *M. tuberculosis* strains H37Rv and CDC1551. The array design is available in BμG@Sbase (Accession No. A-BUGS-31; http://bugs.sgul.ac.uk/A-BUGS-31) and also ArrayExpress (Accession No. A-BUGS-31).

2.3. Whole-genome sequencing and sequence analysis

Library generation and genomic sequencing of the three strains with spoligotypes SB0121, SB0265 and SB0295 was carried out on the Illumina Genome Analyser IIx platform (UCD Conway Institute, University College, Dublin, Ireland). The libraries were constructed as per Tong et al. (2010) with the exception of replacement of the standard Illumina adapters with bar-coded Illumina-compatible adapters as per those detailed by Craig et al. (2008). Library indexing allowed multiplexing of the three samples in a single lane of the Flowcell.

All three genomes, which will be in free access, had theoretical fold coverage of more than 30 times with coverage of the genome sequence of *M. bovis* AF2122/97 of more than 99.8% (including repeat regions). The raw reads were mapped with RoVar (version 30 Nov 2009) against the publicly available genomes of *M. tuberculosis* H37Rv (NC_000962), extracted from the NCBI microbial database on 3 June 2010, *M. bovis* AF2122/97 (NC_002945), *M. bovis* BCG str. Tokyo 172 (NC_012207.1) and *M. bovis* BCG str. Pasteur 1173P2 (NC_008769.1), all three extracted from the NCBI microbial database on 19 May 2010. RoVar (Robust Variant detection in genome sequences using Next Generation Data from various platforms) is available upon request from the authors (https://trac.nbic.nl/rovar/, V.C.L. de Jager, B.A.M. Renckens, R.J. Siezen, and S.A.F.T. van Hijum, unpublished). The "uniquereads" and "coverage" options of RoVar were used to determine insertions and deletions that exceed the read length of 36 bases.

Identified variations were filtered by the following criteria: at least three unique reads cover the region with an identified variation and variations with a low coverage up to six reads were excluded when either forward or reverse reads were missing. More than two perfect matching reads (reads that are identical to the reference sequence) were not tolerated for positions that also show a variation. Variations common to all three strains against the reference genome of *M. bovis* AF2122/97 were collected.

For phylogenetic reconstruction SNPs, but not insertions or deletions, were collected that were identified in 1003 *M. bovis* AF2122/97 genes by RoVar. These 1003 were present in all four reference genomes and did not contain perfect or near-perfect repeats of 30 bases to avoid mismatches of the sequence reads. Only SNPs that were covered by at least seven sequencing reads were gathered. The SNPs were arranged in a SNP matrix and concatenated. The phylogenetic tree was reconstructed using the Neighbour-joining algorithm as implemented in Clustal 2.0.1 (Larkin et al., 2007) and the significance of branch lengths was tested by 1000 times bootstrapping (phyML version 2.4.4, Guindon and Gascuel, 2003). *M. tuberculosis* H37Rv was used to root the tree that was visualised in Dendroscope (version 2.7.4, Huson et al., 2007).

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Table 1
Primer sequences for the screening of single nucleotide polymorphism in genes *ppgK, ureC, fas, rplS, fbiB, dppC, Mb3761c, Mb0489c, Mb0105, Mb1710, Mb0554, gcpE, Mb3697c, Mb3913c, pitB* and *guaA*.

Locus	Forward (5′–3′)	Reverse (5′–3′)	Band size (bp)	Annealing temperature (°C)
Mb2721 (*ppgK*)	GTGAGGGTCATCCACGTCTC	AAGATGTCCGAACTCGGTGT	550	61
Mb1881 (*ureC*)	CACGTGCACTTGATCTGTCC	GAGATGCTCGTCAAGGGTGT	540	61
Mb2553c (*fas*)	GAGATCTGGCGGCAATAAAG	CGGGATAGAAACCGAGTTGG	600	61
Mb2928c (*rplS*)	CAGCGCTTCTCCTTGATCTT	GATTTCACTGCATGCGTACC	520	61
Mb3290 (*fbiB*)	CATACGGCAATGAGTTGGTG	ACCGTGAACATGGTGTGCT	571	61
Mb3688c (*dppC*)	CTCGGTGACGATGAAGATCC	TCCTGCTGATTCTTGTCGTG	572	61
Mb3761c	CCTTGACGTTGAACATGCTG	CCTGAGTTCGTCCGTGAGTT	583	61
Mb0489c	CGTCGACTGAACGGTTTCTT	ACCCGCTAACCGTCTTTCTT	566	61
Mb0105	CTTGTCCCCACTGTGCTTG	GCAGTTCATAGCCGAGGAAG	502	61
Mb1710	AGTCCGCACTGTTGGTCAG	GTCGTCACGCCAGAATAGGT	566	61
Mb0554	GTGCTGGTGGTCGCTAAAG	GTCGTCCACGATGTCGAAGT	570	60
Mb2893c (*gcpE*)	TGGCTTTGCCATACTTCTCC	GCGACCTATGTGGTCTTGGT	593	60
Mb3697c	GACGACGTTAACCGGAAAGA	GTTCTGGCCCTGATGCAC	433	60
Mb3913c	TAACGGCCTGTCTGACTGTG	AACGGTCTGCACATTGTTCC	460	60
Mb2302 (*pitB*)	GACAATGCCAAACACCACAG	TTAGCAAACAGCAGCCAGAG	491	60
Mb3429c (*guaA*)	TCAGCAGTCTTACCGTCCAG	AGCCGGTATGGATGAGTCAC	589	60

2.4. Single nucleotide polymorphism analysis

The SNPs identified in this study (Supplementary Table 2) were assessed by sequencing PCR-amplified fragments of the genes *ppgK*, *ureC*, *fas*, *rplS*, *fbiB*, *dppC*, *Mb3761c*, *Mb0489c*, *Mb0105*, *Mb1710*, *Mb0554*, *gcpE*, *Mb3697c*, *Mb3913c*, *pitB* and *guaA* by cycle sequencing and analysis of reactions on an ABI Prism 373 DNA sequencer (Applied Biosystems). For this purpose, we screened ten Spanish *M. bovis* isolates (Table 2), seven of which had spacer 21 missing in their spoligotype pattern. PCR reactions were carried out in 20 μl total volume using 10 μl of HotStarTaq Master Mix (Qiagen), 0.25 μM of each primer and 2 μl of heat-killed *M. bovis* supernatant. Primers for sequencing SNPs are shown in Table 1.

2.5. SNP screening by PCR-REA

To determine the presence of the SNP in *guaA* a PCR-restriction endonuclease analysis (PCR-REA) was set up and used in the 444 samples. PCR with primers guaA2-F (5′ GTCTTACCGTCCAGCACATCC 3′) and guaA2-R (5′ CAGGTGCAACGCGATTTC 3′) was carried out under the following cycling conditions: initial 95 °C for 15 min followed by 35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 62 °C and 1 min extension at 72 °C and a final extension of 7 min at 72 °C. Subsequently, 8 μl of the amplified product were digested with 5 U of restriction enzyme *Nar*I (New England Biolabs) for 3 h at 37 °C and run on a 2.5% agarose gel at 60 V for 60 min. The absence of the SNP, as in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97, results in two bands of 145 and 34 bp. In case of the presence of the SNP a single band of 179 bp is obtained (Fig. 1). The assay was verified by analysing 52 samples additionally by sequencing with primers as in Table 1.

3. Results

3.1. DNA microarray analysis

The comparison of the four Spanish strains with spoligotypes SB0121, SB0265, SB0295 and SB1337, all lacking spacer 21, to reference strains *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551 and *M. bovis* AF2122/97 confirmed the *M. bovis* absence of the regions of difference RD4–RD13 (Gordon et al., 1999; Brosch et al., 2002) characteristic for *M. bovis*. Furthermore, recently described deletions which define clonal complexes African1 (RDAf1, Müller et al., 2009), African2 (RDAf2, Berg et al., 2011) and European 1 (RDEu1, Smith et al., in press) were found to be intact. The microarray analysis did not lead to identification of possible phylogenetic markers in regions not prone to deletion, such as conserved regions that show no similarity to repetitive DNA and have average GC content.

3.2. SNP selection and analysis

Mapping of the complete genome sequences of the three Spanish strains lacking spacer 21 against publicly available reference genomes of *M. tuberculosis* and *M. bovis* confirmed the absence of spacer 21 from the Direct Repeat (DR) region, but no further large nucleotide deletion was found to be common to the three genomes when compared to NC_002945 (*M. bovis* AF2122/97). However, we identified 108 SNPs (Supplementary Table 2) shared by the three Spanish strains lacking spacer 21 and unique to them. On the assumption that an ideal phylogenetic marker lies within a stable and essential gene, we excluded 25 SNPs that occurred in intergenic regions, in genes with repetitive DNA or in non-conserved genes, which are prone to deletion. We determined the essentiality according to Sassetti et al. (2003), Lamichhane et al. (2003) and Tsolaki et al. (2004) using Tuberculist (http://genolist.pasteur.fr/TubercuList/) and excluded another 57 SNPs in non-essential genes from further analysis. From the remaining 26 SNPs we found 13 SNPs to be located in essential genes and 13 in genes of unknown essentiality. Out of these we selected 16 SNPs randomly from all over the chromosome to further study their suitability as phylogenetic markers for the group of *M. bovis* strains with spacer 21 absent from their spoligotype pattern. After screening of the ten isolates (Table 2) we were able to develop a putative SNP phylogeny for these strains (Fig. 2). Only the SNP in *guaA* was able to distinguish the spacer 21-deleted strains from the strains with spacer 21 present in their spoligotype pattern.

3.3. PCR-REA

The gene *guaA* (*Mb3429c* in *M. bovis* AF2122/97, equivalent to *Rv3396c* in *M. tuberculosis* H37Rv) is a probable guanine monophosphate synthetase (Jungblut et al., 1999) and essential according to Sassetti et al. (2003). The SNP in *guaA* at nucleotide position 3765573 (*M. bovis* AF2122/97) is a synonymous nucleotide change in a triplet encoding alanine (Genbank accession number JF920303). The strains selected for PCR-REA analysis represent each country's *M. bovis* population according to previously published surveys (Haddad et al., 2001; Duarte et al., 2008; Boniotti et al., 2009; Rodriguez et al., 2010) with a special focus on spacer 21-deleted strains (Table 3). PCR-REA targeting the nucleotide change was carried out in all 444 isolates. The presence of this

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Table 2
Results of the sequencing of 16 selected single nucleoide polymorphisms in ten selected *Mycobacterium bovis* strains. Only the SNP in *guaA* differentiates between *M. bovis* strains with and without spacer 21 in their spoligotype pattern.

Strain reference	Spoligotype	Sp21[a]	*ppgK* (C to T)	*ureC* (A to G)	*fas* (G to A)	*rplS* (G to A)	*fbiB* (A to C)	*dppC* (G to A)	*Mb3761c* (G to A)	*Mb00489c* (C to T)	*Mb0105* (C to T)	*Mb1710* (C to T)	*Mb0554* (G to A)	*gcpE* (T to C)	*Mb3697c* (C to G)	*Mb3913c* (T to C)	*pitB* (G to A)	*guaA* (G to A)
M. tuberculosis H37Rv[b]	SIT451	–	C	A	G	G	A	G	G	C	C	C	G	T	C	T	G	G
M. bovis AF2122/97[b]	SB0140	+	C	A	G	G	A	G	G	C	C	C	G	T	C	T	G	G
MI05/02146	SB0120	+	T	A	G	G	A	G	G	C	C	C	N[c]	T	C	T	N[c]	G
MI08/07990	SB0120	+	N[c]	A	A	A	C	A	N[c]	T	T	T	A	C	G	C	A	G
MI07/03177	SB1299	+	C	A	G	G	A	G	G	C	C	C	G	N[c]	G	T	G	G
MI07/14549	SB0121	–	T	G	A	A	C	A	A	T	T	T	A	C	G	C	A	A
MI09/08699	SB0121	–	T	G	A	A	C	A	A	T	T	T	A	C	G	C	A	A
MI08/09244	SB0152	–	T	G	A	A	C	A	A	T	T	T	N[c]	C	G	C	A	A
MI08/12026	SB1263	–	T	G	A	A	C	A	A	T	T	T	A	C	G	C	A	A
MI05/02739	SB1343	–	T	G	A	A	C	A	A	T	T	T	A	C	G	C	A	A
MI05/04493	SB1350	–	T	G	A	A	C	A	A	T	T	T	N[c]	C	G	C	A	A
MI06/04185	SB1380	–	T	G	A	A	C	A	A	T	T	T	A	C	G	C	A	A

[a] Spacer 21 present (+) or deleted (–) in the spoligotype pattern.
[b] Reference strains.
[c] Not amplifiable.

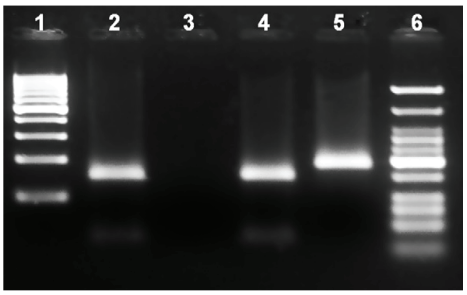

Fig. 1. PCR-restriction enzyme analysis of *guaA* with *Nar*I run on a 2.5% agarose gel. The absence of the SNP results in two bands of 145 and 34 bp, while the presence of the SNP leads to a single band of 179 bp. Lane 1, molecular ladder 100 bp (Biotools); lane 2, *Mycobacterium tuberculosis* H37Rv; lane 3, negative control; lane 4, *M. bovis* AF2122/97; lane 5, MI06/05041 (Eu2); lane 6, low molecular weight marker 25–766 bp (New England Biolabs).

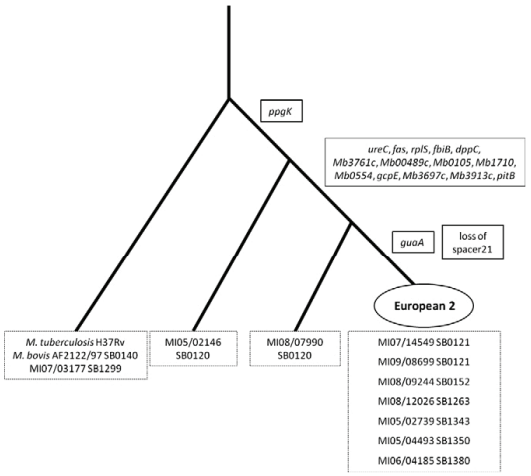

Fig. 2. Schematic showing the evolutionary relationship of ten Spanish isolates and *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 based on the screening of 16 SNPs in *ppgK*, *ureC*, *fas*, *rplS*, *fbiB*, *dppC*, *Mb3761c*, *Mb0489c*, *Mb0105*, *Mb1710*, *Mb0554*, *gcpE*, *Mb3697c*, *Mb3913c*, *pitB* and *guaA*. This representation of the relationship between the strains does not imply a molecular clock.

SNP was confirmed in 138 (69%) of the Spanish and in 37 (77%) of the Portuguese isolates. Of the French isolates 40 (28%) showed the SNP in *guaA* while only two (4%) of the Italian strains were mutated. All the *guaA* mutated isolates lacked spacer 21 in their spoligotype pattern. In 11 isolates with spacer 21 missing [spoligotypes SB1257 ($n = 1$) from Spain; SB0840 ($n = 1$), SB0881 ($n = 2$), SB0894 ($n = 1$), SB0895 ($n = 1$), SB0982 ($n = 1$), SB1005 ($n = 1$) from France; SB1305 ($n = 1$) and SB1561 ($n = 2$) from Italy)] *guaA* was not mutated. For five isolates no results could be obtained. The SNP was absent from all other isolates (Supplementary Table 1) and from *M. tuberculosis* H37Rv, *M. bovis* AF2122/97, *M. bovis* CHAD491 (African 1 clonal complex; Müller et al., 2009) and BTB1087 (African 2 clonal complex; Berg et al., 2011).

4. Discussion

In this study we report the identification of a clonal complex of *M. bovis*, named European 2 (Eu2), prevalent in Western

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Table 3
Mycobacterium bovis strain selection from each country in comparison to the respective population surveys and predicted frequency of the European 2 (Eu2) clonal complex in each country.

Country	Reference	Number of isolates	Strains with spacer 21 missing (%)	Number of isolates in this study	Isolates with spacer 21 missing included in this study (%)	Predicted maximum of Eu2 strains (%)[a]
Spain	Rodriguez et al. (2010)	6215	67	201	70	66.5
Portugal	Duarte et al. (2008)	283	74	48	79.2	74
France	Haddad et al. (2001)	1349	25	145	32.4	21.3
Italy	Boniotti et al. (2009)	1560	4	50	10	1.6

[a] Strains lacking spacer 21 in their spoligotype pattern that are not members of the Eu2 clonal complex reduced the maximum percentage of Eu2 isolates in each country.

continental Europe and we show the relationship among neighbouring countries regarding the demography of the pathogen. This finding was based on surveys of the *M. bovis* population using DVR-spoligotyping in Spain, Portugal, Italy and France and on the analysis of representative collections of *M. bovis* isolates from these countries.

The availability of molecular techniques has enabled the characterisation of *M. bovis* isolates for epidemiological studies. Most of these efforts have been devoted to studies of transmission of the pathogen, for example between wildlife and domestic animals sharing the same habitat (Romero et al., 2008; Richomme et al., 2010). Recently, these tools have also been applied to population genetics to define the clonal complexes Af1, Af2 and Eu1 (Müller et al., 2009; Berg et al., 2011; Smith et al., in press). These studies identified deletions, RDAf1, RDAf2 and RDEu1, that are unique and define clonal complexes which are geographically restricted and marked by specific spoligotype signatures [absence of spacer 30 (Af1), absence of spacers 3–7 (Af2), absence of spacer 11 (Eu1)]. Associations between spoligotype patterns and certain lineages have also been observed for subclones of *M. tuberculosis* (Kato-Maeda et al., 2011). The suitability of a deletion as a phylogenetic marker depends on the stability and essentiality of the affected genomic region; deletions in regions with a high mutation rate which may be prone to deletion, such as regions with nonessential genes or with repetitive DNA do not offer ideal targets. In this study we were unable to identify a phylogenetically informative deletion by microarray, so that whole-genome sequencing and determination of single nucleotide polymorphism was needed to further define this clonal complex.

The 108 SNPs common to the three strains lacking spacer 21 distinguished them from *M. tuberculosis* H37Rv, *M. bovis* AF2122/97, *M. bovis* BCG Pasteur and *M. bovis* BCG Tokyo. Furthermore, the mutation at *guaA* was not observed in reference strains of previously described clonal complexes Af1, Af2 and Eu1, which suggests that these form different clusters from the Eu2 clonal complex; nevertheless, a comparison of the three sequenced strains to the whole genome sequences of Af1 and Af2 strains would be interesting to elucidate the differences between the clonal complexes at SNP level.

The Eu2 lineage is present in four countries in Western continental Europe albeit at different frequencies. According to the frequency of strains with spacer 21 lacking in their spoligotype, the presence of strains belonging to the Eu2 clonal complex is predicted to be low (1.6%) in Italy, moderate (20.4%) in France and highest in Spain (66%) and Portugal (74%) (Table 3 and Fig. 3). In the British Isles, the presence of spacer 21-deleted spoligotypes is rare and 99% of the strains are deleted for a phylogenetically informative deletion (RDEu1) and therefore members of the Eu1 clonal complex (Smith et al., in press). Hence, we conclude that strains of the Eu2 clonal complex are rare or absent from the British Isles (Fig. 3).

The most recent common ancestor of the Eu2 clonal complex already featured the defining marker of the Eu2 clonal complex (SNP in

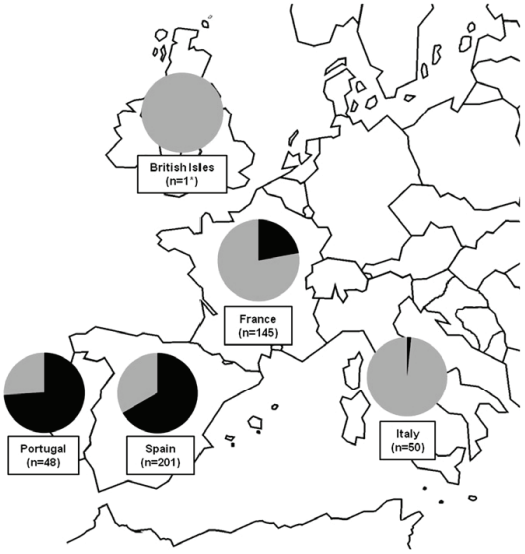

Fig. 3. Map of Western Europe showing the distribution of the Eu2 clonal complex of *M. bovis* in Spain, Portugal, France, Italy and the British Isles and the number of *M. bovis* isolates analysed from each country. The pie charts show the predicted proportion of strains that are members of the Eu2 clonal complex (black = Eu2, grey = others). Strains of Eu2 dominate in the Iberian Peninsula, but are only present at low frequency in France and Italy and rare in the British Isles (*reference strain *M. bovis* AF2122/97 was used as prototype of the British and Irish *M. bovis* population).

guaA) and probably also lacked spacer 21 in its spoligotype pattern. Since the evolution of the DR region is unidirectional, occurring by single spacer deletions or loss of contiguous spacer sequences (Fang et al., 1998; van Embden et al., 2000), and due to the strict clonality of the *M. tuberculosis* complex, genetic features are passed on to all the descendants (Smith et al., 2006), all the Eu2 members are marked by the SNP in *guaA* and absence of spacer 21. However, the association between the spacer 21 deletion and the SNP in *guaA* is not exclusive and loss of spacer 21 can occur in non-Eu2 isolates caused by homoplasy. This is highlighted by the finding of 11 isolates with spacer 21 absent from their spoligotype patterns which were not mutated at *guaA*. The emergence of identical spoligotypes in unrelated strain lineages is possible as a result of convergent evolution (Warren et al., 2002) and the spoligotype signature should only be used as an indicator but not a definition of a clonal complex (Smith et al., in press).

We hypothesise that a group of strains of *M. bovis* entered the Iberian Peninsula on the landroute through the Pyrenees or on maritime routes, and that these founder strains subsequently

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underwent expansion, colonising cattle naïve to bovine tuberculosis. More conclusions regarding Eu2 strains and whether they are descendants or ancestors of the Italian or French *M. bovis* population could be drawn from sequencing genomes of French and Italian strains.

Members of the Eu2 clonal complex are defined by the mutation in *guaA* while the loss of spacer 21 can be used as a preliminary screen to identify its members. Possible Eu2 members are also present in countries in other continents (Zumarraga et al., 1999; Michel et al., 2008) where they possibly have been introduced through international trade during the last centuries. It would be intriguing to analyse isolates from those countries in order to further estimate the presence of the Eu2 clonal complex at a worldwide level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.09.004.

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Supplementary Table 1 contains the strain information of the 444 M. bovis isolates used in this study and the corresponding nucleotide at position 3765573 (M. bovis AF2122/97) and is available at doi:10.1016/j.meegid.2011.09.004. Supplementary Table 2 resumes the 108 single nucleotide polymorphisms identified by whole genome sequencing and is shown below and also available at doi:10.1016/j.meegid.2011.09.004.

Supplementary Table 2. The 108 single nucleotide polymorphisms that are common and unique to the three Spanish strains and their position regarding *Mycobacterium bovis* AF2122/97 (Supplementary Table 2).

SNP number	Position in *M. bovis* AF2122/97 NC_002945	Reference base	Reads base	Locus description
1	1057	A	G	*Mb0001* (*dnaA*)
2	31613	C	G	*Mb0029*
3	37913	G	C	*Mb0036* (*fadD34*)
4	98797	T	G	*Mb0093*
5	118504	C	T	*Mb0105**
6	222939	A	G	*Mb0197*
7	224919	A	G	*Mb0199c*
8	228816	G	A	*Mb0200*
9	239255	C	T	*Mb0208c* (*mmpL11*)
10	290174	C	T	upstream *Mb0248c* (*fadA2*)
11	292007	T	C	190 bp downstream of *Mb0249* (*fadA2*) ; 116 bp downstream of *Mb0250c* (*fadE5*)
12	311930	C	A	*Mb0267c* (*narK3*)
13	316798	T	C	*Mb0272c* (*oplA*)
14	445873	C	T	*Mb0374c*
15	541959	T	C	*Mb0458c* (*mmpL4*)
16	569303	C	T	*Mb0489c**
17	616227	G	A	*Mb0537* (*hemL*)
18	627327	C	T	*Mb0548c* (*menA*)
19	633828	G	A	*Mb0554**
20	637122	T	C	3 bp upstream of *Mb0557c* ; 57 bp downstream of *Mb0558c*
21	913005	C	T	*Mb0842* (*mshD*)
22	1027438	G	A	156 bp upstream of *Mb0944c* ; 132 bp upstream of *Mb0945*
23	1140712	G	C	*Mb1048* (*mfd*)
24	1160900	C	T	20 bp upstream of *Mb1065c* ; 91 bp downstream of *Mb1066c* (*esxI*)
25	1196410	C	A	7 bp upstream of *Mb1099c* (*echA8*) ; 5 bp downstream of *Mb1100c* (*echA9*)

* Single nucleotide polymorphisms chosen for the screening for a suitable phylogenetical marker for strains with spacer 21 missing in their spoligotype pattern.

SNP number	Position in *M. bovis* AF2122/97 NC_002945	Reference base	Reads base	Locus description
26	1271546	C	T	*Mb1175* (*mcr*)
27	1280770	C	A	*Mb1182c*
28	1307633	G	T	*Mb1208c* (*fadH*)
29	1318941	T	A	*Mb1213* (*pks3*)
30	1371731	T	C	*Mb1259c*
31	1422256	G	A	60 bp upstream of *Mb1304c* ; 87 bp upstream of *Mb1305* (*lprB*)
32	1433174	C	T	*Mb1312c* (*oppD*)
33	1442426	A	G	*Mb1321c*
34	1446945	T	G	*Mb1325* (*lysA*)
35	1484080	G	T	*Mb1358* (*fadA4*)
36	1529566	A	G	*Mb1395* (*Rv1360*)
37	1530353	T	C	*Mb1395* (*Rv1360*)
38	1532417	C	T	*Mb1397c*
39	1618701	T	C	*Mb1478c*
40	1691313	C	T	*Mb1542c*
41	1756736	T	C	*Mb1591c* (*treX*)
42	1763235	T	G	*Mb1597* (*bioD*)
43	1772279	C	T	*Mb1611c*
44	1779047	C	T	*Mb1618c*
45	1791812	C	T	*Mb1633* (*chaA*)
46	1812300	C	T	*Mb1651c* (*cya*)
47	1888376	G	A	*Mb1705*
48	1895129	C	T	*Mb1710**
49	1955471	C	T	*Mb1772* (*pknE*)
50	2001139	C	T	*Mb1806* (*cyp144*)
51	2088783	A	G	*Mb1881* (*ureC*)*
52	2116222	T	C	*Mb1908*, Similar to 5' end of *Rv1877*
53	2234912	A	G	11 bp downstream of *Mb2029* (*otsB1*) ; 88 bp downstream of *Mb2030c* (*fdxA*)

* Single nucleotide polymorphisms chosen for the screening for a suitable phylogenetical marker for strains with spacer 21 missing in their spoligotype pattern.

SNP number	Position in *M. bovis* AF2122/97 NC_002945	Reference base	Reads base	Locus description
54	2238055	G	A	*Mb2034c*
55	2326429	A	G	*Mb2115* (*pknJ*)
56	2371178	A	C	*Mb2153c*
57	2386636	T	C	*Mb2171c*
58	2450875	G	A	*Mb2230* (*cobT*)
59	2530991	G	A	*Mb2302* (*pitB*)*
60	2594136	A	G	*Mb2367* (*mmpL9a*)
61	2600511	G	A	*Mb2373c* (*dgt*)
62	2642159	G	A	*Mb2404c* (*mbtB*)
63	2670247	G	A	*Mb2427c* (*lepA*)
64	2741995	A	G	*Mb2498* (*aglA*)
65	2773202	G	A	169 bp upstream of *Mb2518c* (PE_PGRS43a) ; 260 bp upstream of *Mb2519*
66	2815669	G	A	*Mb2553c* (*fas*)*
67	2858835	C	T	*Mb2597*
68	2930433	G	A	*Mb2669*
69	2931059	G	A	*Mb2670* (*dedA*)
70	2973577	C	T	*Mb2721* (*ppgK*)*
71	2978818	G	A	*Mb2728*
72	3000936	C	T	*Mb2750*
73	3085838	G	A	*Mb2846c*
74	3113540	C	T	*Mb2873c* (*cobB*)
75	3137044	T	C	*Mb2893c* (*gcpE*)*
76	3143492	G	A	42 bp downstream of *Mb2899* (*dipZ*) ; 54 bp upstream of *Mb2900* (*mpb70*)
77	3149904	A	G	231 bp upstream of *Mb2907c* (*pyrH*) ; 4 bp upstream of *Mb2908*
78	3170638	G	A	*Mb2928c* (*rplS*)*
79	3181043	C	T	*Mb2939c*
80	3223228	G	A	*Mb2959* (*ppsD*)
81	3265346	G	A	113 bp upstream of *Mb2979c* ; 10 bp upstream of *Mb2980*

* Single nucleotide polymorphisms chosen for the screening for a suitable phylogenetical marker for strains with spacer 21 missing in their spoligotype pattern.

SNP number	Position in *M. bovis* AF2122/97 NC_002945	Reference base	Reads base	Locus description
82	3279148	C	T	*Mb2991c* (*pca*)
83	3287646	G	A	*Mb2999c*
84	3337355	C	T	*Mb3048c* (PE29)
85	3343529	G	C	155 bp upstream of *Mb3053c* ; 106 bp downstream of *Mb3054c* (*fixB*)
86	3386850	C	G	53 bp upstream of *Mb3091c* ; 84 bp upstream of *Mb3092* (*mmr*)
87	3444629	A	G	*Mb3148c* (PPE49)
88	3456790	C	C	539 bp downstream of *Mb3159* (PPE50) ; 113 bp upstream of *Mb3160* (PPE51)
89	3512801	T	C	*Mb3213c*
90	3532002	T	C	*Mb3227c*
91	3597078	A	C	*Mb3290* (*fbiB*)*
92	3677456	C	T	*Mb3366c*
93	3738672	C	T	*Mb3406*
94	3765573	G	A	*Mb3429c* (*guaA*)*
95	3829395	C	T	*Mb3492*
96	3859018	C	T	*Mb3525c* (*lprN*)
97	3934159	C	A	*Mb3582*
98	3963224	T	C	*Mb3608*
99	4037153	C	A	*Mb3685*
100	4041523	G	A	*Mb3688c* (*dppC*)*
101	4052166	G	C	*Mb3697c**
102	4065884	C	T	*Mb3710c* (*cyp137*)
103	4122931	G	A	*Mb3761c**
104	4200820	C	T	182 bp upstream of *Mb3832c* ; 16 bp downstream of *Mb3833c* (*fbpD*)
105	4241021	C	T	*Mb3859c*
106	4300689	T	C	*Mb3913c**
107	4315851	G	A	*Mb3925c*
108	4324278	C	G	*Mb3933c*

* Single nucleotide polymorphisms chosen for the screening for a suitable phylogenetical marker for strains with spacer 21 missing in their spoligotype pattern.

IV.2. The African 2 clonal complex of *M. bovis*

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African 2, a Clonal Complex of *Mycobacterium bovis* Epidemiologically Important in East Africa▽†

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We have identified a clonal complex of *Mycobacterium bovis* isolated at high frequency from cattle in Uganda, Burundi, Tanzania, and Ethiopia. We have named this related group of *M. bovis* strains the African 2 (Af2) clonal complex of *M. bovis*. Af2 strains are defined by a specific chromosomal deletion (RDAf2) and can be identified by the absence of spacers 3 to 7 in their spoligotype patterns. Deletion analysis of *M. bovis* isolates from Algeria, Mali, Chad, Nigeria, Cameroon, South Africa, and Mozambique did not identify any strains of the Af2 clonal complex, suggesting that this clonal complex of *M. bovis* is localized in East Africa. The specific spoligotype pattern of the Af2 clonal complex was rarely identified among isolates from outside Africa, and the few isolates that were found and tested were intact at the RDAf2 locus. We conclude that the Af2 clonal complex is localized to cattle in East Africa. We found that strains of the Af2 clonal complex of *M. bovis* have, in general, four or more copies of the insertion sequence IS*6110*, in contrast to the majority of *M. bovis* strains isolated from cattle, which are thought to carry only one or a few copies.

Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, is mainly a disease of cattle, but it is also a zoonosis infecting humans. Bovine TB has been eradicated in Australia and many European countries; however, it is still believed to be common among cattle throughout the rest of the world. On the African continent, information on the prevalence of bovine TB is scarce and control programs are in place in only a few countries (6, 16). However, a number of reports suggest that the disease is widely spread over the African continent and highly prevalent in several countries (8, 21, 38, 42, 51), with infection present mainly in cattle but also in wildlife (39).

M. bovis is one of seven species constituting the *Mycobacterium tuberculosis* complex, which includes *M. tuberculosis*, one of the most devastating bacterial pathogens of humans. There is little or no exchange of chromosomal DNA between cells from the *M. tuberculosis* complex, making this group of bacteria highly clonal (14, 30, 53–54). In a strictly clonal population, any mutation present in an ancestral strain will be present in all

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its descendants and can be used to identify clonal complexes. A series of deletions (regions of difference [RD]) within the *M. tuberculosis* complex have been used to identify phylogenetic relationships between members of the *M. tuberculosis* complex (11), and for *M. tuberculosis*, different lineages and sublineages have also been characterized by specific deletions (25, 60). In a similar manner, we are exploring the relationships between lineages of *M. bovis* that dominate in different geographical regions around the world.

Spoligotyping, a PCR and hybridization technique, is the most common genotyping technique for strains of the *M. tuberculosis* complex and assays polymorphism in 43 unique spacer sequences found in the direct repeat (DR) region (36, 61). Each spoligotype pattern from strains of the animal-adapted lineage of the *M. tuberculosis* complex is given a unique identifier by www.Mbovis.org. Several studies of the DR region in closely related strains of *M. tuberculosis* have concluded that the evolutionary trend of this region is primarily by loss of single or multiple contiguous spacers (23, 29, 63); duplication of direct variable repeat (DVR) units or point mutations in spacer sequences were found to be rare. Although the absence of specific spacers, or groups of spacers, in a spoligotype pattern can be indicative of a closely related group of strains (clonal complex), spacers are frequently lost independently in different lineages (homoplasy). Furthermore, the interpretation of specific spacer loss, such as that of spacers 3 to 7 in the strains described in this article, can be ambiguous if adjacent spacers in the spoligotype pattern are also deleted.

Recently a clonal complex of *M. bovis*, called African 1 (Af1) (41), that is highly prevalent in several countries of west-central Africa has been identified. In this article, we identify a second *M. bovis* clonal complex common in East Africa and name this group of strains the African 2 (Af2) clonal complex of *M. bovis*.

MATERIALS AND METHODS

Bacterial strains. The majority of all *M. bovis* strains analyzed in this study were isolated from cattle and are described in more detail in the supplemental material. One hundred twenty strains were collected from six abattoirs in Ethiopia during 2006 to 2008 (8); nine strains were collected at an abattoir in Kampala from cattle originating from seven districts in Uganda (5); ten strains were collected from three sites in or close to the capital Bujumbura in Burundi (48); and fourteen strains were collected from cattle at a Morogoro slaughterhouse in Tanzania (41). Additional population samples of *M. bovis* isolated from cattle from South Africa ($n = 22$) (40]), Chad ($n = 5$) (35]), Mali ($n = 20$) (42), Cameroon ($n = 3$), Nigeria ($n = 5$), Mozambique ($n = 14$), Algeria ($n = 17$) (51), Italy ($n = 93$) (10), and Spain ($n = 20$) (49]) were analyzed (see the supplemental material). Also, two strains of *M. bovis* from humans, isolated in Uganda and Sweden, were further investigated for this study.

All isolates were characterized by spoligotyping, and the majority were also deletion typed for regions RD4 and RDAf2. Selected *M. bovis* isolates were subjected to variable-number tandem repeat (VNTR) typing (24) and RDAf1 deletion typing (41) (see the supplemental material). Isolates of *M. tuberculosis* $H_{37}Rv$ and *M. bovis* AF2122/97 were used as controls.

Spoligotyping, VNTR typing, and microarray analysis. Strains were spoligotyped according to the method of Kamerbeek et al. (36) with minor modifications (12), and the exact tandem repeat (ETR) loci ETR-A to ETR-F were VNTR typed as previously described (12, 24). The VNTR types are displayed as a series of six integers representing the deduced number of repeats present at each locus. All VNTR typing was performed at the VLA, Weybridge, United Kingdom.

For microarray analysis, two isolates (no. BTB0691 and BTB1091; see the supplemental material) were selected from the Ethiopian *M. bovis* collection. Both isolates lacked spacers 3 to 7 in their spoligotype pattern. Approximately 1 to 2 μg genomic DNA was purified, and deletions were identified by microarray analysis using previously published methods (26). Deletions found in regions associated with repetitive elements and insertion sequences, which are known to be prone to deletion events, were disregarded in this study.

Deletion typing. The identification of a strain as *M. bovis* was on the basis of spoligotype signature (56) and growth characteristics; many of the isolates from Uganda, Burundi, Tanzania, and Ethiopia were confirmed as *M. bovis* by deletion typing of the RD4 region (11). The status of the RDAf2 region (deleted or intact) was assessed by multiplex PCR with a set of three primers (primer set Af2): two primers targeting the flanking regions of RDAf2 (RDAf2_Fw, 5′-ACTGGACCGGCAACGACCTGG, RDAf2_Rev, 5′-CGGGTGACCGTGAACTGCGAC) and one primer hybridizing with the internal region of RDAf2 (RDAf2_IntRev, 5′-CGGATCGCGGTGATCGTCGA). A PCR product of 458 bp (RDAf2 intact) or 707 bp (RDAf2 deleted) was identified by agarose gel electrophoresis. Each PCR mixture contained 1 μl of supernatant of heat-killed mycobacterial cells, a final concentration of 1× HotStartTaq master mix (Qiagen), 1 μM primers RDAf2_FW, RDAf2_Rev, and RDAf2_IntRev, and sterile distilled water to a final volume of 20 μl. Thermal cycling was performed with an initial denaturation step of 15 min at 96°C, 35 cycles of 30 s at 96°C, 30 s at 55°C, and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. PCR products were separated on a 1% agarose gel. Isolates subjected to RDAf1 typing were examined according to a previously described PCR protocol (41).

IS*6110* RFLP typing. Genomic DNA was purified from selected *M. bovis* strains (66), and approximately 2 μg DNA was used for IS*6110* restriction fragment length polymorphism (RFLP) analysis according to the internationally standardized protocol (62). In short, DNA was digested with the restriction endonuclease PvuII, separated by agarose gel electrophoresis, and transferred to a nylon membrane by Southern blotting. The membrane was hybridized with a probe targeting the right-hand site of the IS*6110* element (62, 65) and subsequently with a 36-bp oligonucleotide targeting the direct repeat region (65). The probes were labeled using the enhanced chemiluminescence detection system (ECL; Amersham). The IS*6110* RFLP patterns were analyzed by using the BioNumerics software program (Applied Maths, Sint Martens-Latum, Belgium), and the dendrogram was prepared by using the Dice coefficient and unweighted-pair group method using average linkages (UPGMA).

Nucleotide sequence accession numbers. The RDAf2 deletion junctions of 10 strains of the Af2 clonal complex from five countries were sequenced using standard methods. The isolate name, country of origin, and GenBank accession numbers for the sequences surrounding the RDAf2 deletion junctions are as follows: BTB0890, Ethiopia, GU004183; BTB1067, Ethiopia, GU004182; BTB1474, Ethiopia, GU004184; JN03, Uganda, GU004178; JN58, Uganda, GU004179; SEA199701128, Somalia, GU004185; 940130, Burundi, GU004186; 940439, Burundi, GU004187; 11, Tanzania, GU004180; and B3, Tanzania, GU004181 (see the supplemental material).

RESULTS

Isolates with spacers 3 to 7 absent. An extensive slaughterhouse study in Ethiopia of 58 *M. bovis* strains isolated from six abattoirs dispersed throughout the country showed that many isolates lacked spacers 3 to 7 in their spoligotype pattern, in addition to the absence of spacers 9, 16, and 39 to 43 (8). We supplemented this sample with an additional 62 isolates from the same abattoirs and found that over 75% ($n = 91$; total = 120) of these Ethiopian isolates had spoligotype patterns that were missing spacers 3 to 7 (Table 1).

Furthermore, three separate spoligotype surveys of bovine TB in Ethiopian cattle from Addis Ababa and central/southern Ethiopia showed similar results: over 80% of strains had spacers 3 to 7 deleted (2, 9, 59).

From Uganda, which is situated close to Ethiopia, it was recently shown that six of nine *M. bovis* isolates from cattle originating from seven districts in both the northwest and southern parts of the country also had spacers 3 to 7 missing in their spoligotype pattern (5). The absence of spacers 3 to 7 in Ugandan isolates was supported by a further spoligotype survey of 19 *M. bovis* isolates sampled from cattle from similar regions of the country (44).

To further identify the clonal complexes of bovine TB in

TABLE 1. Spoligotype patterns of *M. bovis* strains isolated in four east African countries[a]

Country	Pattern designation[b]	Spoligotype pattern[c]	Frequency [no. (%) of strains]	RDAf2
Uganda	SB1407	11000001011111101100001111111111111111100000	3 (33.3)	Deleted
	SB1405	01011001011111101111111111111111111101100000	2 (22.2)	Intact
	SB1406	01011001011111101111111111111111011101100000	1 (11.1)	Intact
	SB0133	**11000001011111101111111111111111111111100000**	1 (11.1)	Deleted
	SB1404	11000001011110001111111111111111111111100000	1 (11.1)	Deleted
	SB1408	11000001011110001100001111111111111101100000	1 (11.1)	Deleted
Total			9 (100.0)	
Burundi	SB0303	11000001011101101111111111111111111111100000	5 (50.0)	Deleted
	SB1388	11000001011111101110111111111111111111100000	4 (40.0)	Deleted
	SB0304	11000001011111101111000011111111111111100000	1 (10.0)	Deleted
Total			10 (100.0)	
Tanzania	**SB0133**	**11000001011111101111111111111111111111100000**	9 (64.3)	Deleted
	SB0425	01010111010001101111111111110000001011000000	4 (28.6)	Intact
	SB1446	11000001010111101111111111111111111111100000	1 (7.1)	Deleted
Total			14 (100.0)	
Ethiopia	SB1176	11000001011111101111111000100000000000100000	59 (51.6)	Deleted
	SB1476	11011111011111101111111111111111100000100000	21 (16.7)	Intact
	SB0133	**11000001011111101111111111111111111111100000**	18 (14.3)	Deleted
	SB1477	10000001011111101111111111111111011111100000	8 (6.3)	Deleted
	SB0134	11000111011111101111111111111111111111100000	6 (4.8)	Intact
	SB0120	11011111011111101111111111111111111111100000	2 (1.6)	Intact
	SB1942	11000001011111101111111111111111111001100000	2 (1.6)	Deleted
	SB1488	11000001011111101111111110011111111111100000	1 (0.8)	Deleted
	SB1489	11000001011111101111111111111111010111100000	1 (0.8)	Deleted
	SB1941	00000001011111101111111111111111110111100000	1 (0.8)	Deleted
	SB0303	11000001011101101111111111111111111111100000	1 (0.8)	Deleted
Total			120 (100.0)	

[a] The presumed ancestral spoligotype pattern of the Af2 clonal complex is shown in bold.
[b] International names for these spoligotype patterns were assigned by Mbovis.org (http://www.Mbovis.org).
[c] The spoligotype pattern is shown as a series of 43 ones and zeros, corresponding to spacers 1 to 43 in spoligotyping, with 1 representing hybridization to the spacer and 0 representing the absence of hybridization.

East Africa, we spoligotyped *M. bovis* strains previously collected from cattle in Burundi and Tanzania (41, 48). All isolates from Burundi (originating at the city of Bujumbura and nearby in the western parts of the country; $n = 10$) and 10 out of 14 isolates from cattle sampled at a Morogoro slaughterhouse in south-central Tanzania had spacers 3 to 7 missing in their spoligotype patterns (Table 1).

In total, 117 out of 153 (76%) of *M. bovis* isolates from these four east African countries had spoligotype patterns missing spacers 3 to 7, and we concluded that, in a manner similar to that of the African 1 clonal complex in west-central Africa (41), these isolates may represent a clonal complex of bovine TB descended from an ancestral cell in which spacers 3 to 7 had been deleted. The commonest spoligotype pattern in this data set was SB0133 (Table 2), which is similar to that of *M. bovis* BCG (SB0120, which lacks spacers 3, 9, 16, and 39 to 43), with the additional loss of spacers 4 to 7. Spoligotype pattern SB0133 was found at high frequency in the Tanzanian and Ethiopian samples and in a low number in Uganda and was absent from the small sample of strains from Burundi (Table 1).

Identification of a specific deletion. To identify a phylogenetically informative deletion for the east African *M. bovis* strains lacking spacers 3 to 7, two Ethiopian isolates of spoligotype SB0133, which represents the most complete spoligotype pattern lacking spacers 3 to 7, were tested by microarray analysis using an *M. tuberculosis-M. bovis* composite amplicon microarray. This analysis showed that these two strains were deleted for all regions that are commonly missing in strains of *M. bovis*, including RD4 (11). Region RDAf1, which is deleted

TABLE 2. Definition and summary of characteristics of the Af2 clonal complex of *M. bovis*

Category	Description
Definition	Presence of deletion RDAf2 (14.1 kb between Mb0599 and Mb0610)
Spoligotype marker	Absence of spacers 3 to 7
Spoligotype signature[a]	11000001011111101111111111111111111111100000 (SB0133)
Distribution	At high frequency in East Africa (Uganda, Burundi, Tanzania, and Ethiopia)
IS*6110* copy no.	4 or more copies (infrequently less than 4)

[a] The spoligotype signature represents the assumed spoligotype pattern in the progenitor strain of this clonal complex and is shown as a series of 43 ones and zeros corresponding to spacers 1 to 43 in spoligotyping, with "1" representing hybridization to the spacer and "0" representing absence of hybridization. The international name for this spoligotype pattern was assigned by Mbovis.org (http://www.mbovis.org).

in members of the African 1 clonal complex of *M. bovis*, present at high frequency in several countries in west-central Africa, was intact, showing that these strains were not members of the African 1 clonal complex (41), as was a region called RDEu1, which is associated with isolates from cattle originating in Great Britain (unpublished data). However, we identified a unique region of chromosomal DNA of approximately 14 kb that was deleted in both Ethiopian isolates. The endpoints of this deletion were characterized by nucleotide sequencing and compared to the whole genome sequence of *M. bovis* AF2122/97 (27). We determined that 14,094 bp were deleted, and to our knowledge, this deletion has not previously been described. We named this deletion Region of Difference African 2 (RDAf2).

The RDAf2 deletion removed the entire coding sequences of 10 genes from Mb0600c to Mb0609 and parts of Mb0599 and Mb0610 (corresponding to the genes Rv0585c to Rv0593 and parts of Rv0584 and Rv0594 in *M. tuberculosis* H_{37}Rv). The regions surrounding the RDAf2 deletion junction in the genome of the sequenced strain *M. bovis* AF2122/97 showed no evidence of the common *M. tuberculosis* complex insertion sequences or repetitive DNA and were not GC rich. No significant inverted or direct repeats could be identified at either side of the deletion junctions in the RDAf2 region of the AF2122/97 chromosome sequence. We therefore concluded that this region of the chromosome is not prone to generate homoplastic deletions and hence the RDAf2 deletion could be a suitable phylogenetic marker to identify strains of a clonal complex of *M. bovis* strains which descended from an ancestral cell in which RDAf2 was deleted in addition to spacers 3 to 7 in the spoligotype pattern.

Distribution of RDAf2 among cattle isolates in East Africa. To rapidly identify strains with the RDAf2 deletion, we developed a simple PCR method using two primers targeting both flanking regions of RDAf2 and one primer targeting an RDAf2 internal sequence. All 120 *M. bovis* isolates from cattle from Ethiopia were tested by this deletion assay, and 91 of these were deleted for RDAf2. Furthermore, we tested samples of *M. bovis* collected from cattle from Uganda (5), Burundi (48), and Tanzania (41) for the status of the RDAf2 region. The RDAf2 region was deleted in 6 out of 9 isolates from Uganda, in all isolates sampled from Burundi ($n = 10$), and in 10 of the 14 isolates from Tanzania (Table 1). All strains in these samples and throughout this article that were deleted for RDAf2 were also missing spacers 3 to 7 in their spoligotype pattern.

To provide supportive evidence that the RDAf2 deletion was identical by descent, we sequenced across the RDAf2 deletion junction in nine *M. bovis* isolates of different spoligotypes (at least two from each of the four east African countries). The RDAf2 deletion endpoints were the same in all nine strains, suggesting that in these strains the RDAf2 deletion is identical by descent.

We concluded that a clonal complex of *M. bovis* strains, defined by the deletion RDAf2 and marked by the loss of spoligotype spacers 3 to 7, was present at high frequency in Uganda, Burundi, Tanzania, and Ethiopia (Table 2). We named this *M. bovis* clonal complex African 2.

Af2 in west-central Africa. The *M. bovis* Af1 clonal complex has previously been identified at high frequency in samples of strains from Mali, Nigeria, Chad, and Cameroon (41). In a manner similar to that of Af2, described here, all isolates of the Af1 clonal complex have a specific deletion, RDAf1, and have spacer 30 missing in their spoligotype. To determine the phylogenetic relationship between Af1 and Af2 strains, a selection of isolates which represent the population of Af1 strains present in each of these west-central African countries were deletion typed for the RDAf2 deletion. All Af1 strains from Mali ($n = 13$), Nigeria ($n = 5$), Chad ($n = 5$), and Cameroon ($n = 3$) were intact for RDAf2 (see the supplemental material). Reciprocal deletion analysis showed that strains of Af2 are not deleted for RDAf1 ($n = 27$), and we concluded that Af1 and Af2 were phylogenetically distinct. We also performed RDAf2 deletion typing of seven strains from Mali of spoligotypes SB0134 and SB0991; these patterns represent non-Af1 isolates found in that country (42); these strains were intact at the RDAf2 and RDAf1 loci (41). We concluded, based on the dominance of the Af1 clonal complex in west-central Africa (53), that the Af2 clonal complex was rare or absent from Mali, Nigeria, Chad, and Cameroon.

Af2 elsewhere in Africa. Spoligotype surveys of *M. bovis* from Algeria, Zambia, and South Africa suggest that strains with spacers 3 to 7 deleted are absent or present at low frequency in these countries (40, 43, 47, 51). Furthermore, a spoligotype survey of over 100 strains from Madagascar did not disclose any patterns with spacers 3 to 7 missing (47). To determine the prevalence of Af2 in other African countries, we RDAf2 deletion typed samples representing previously published spoligotype surveys of *M. bovis* from Algeria ($n = 17$) (51) and South Africa ($n = 20$) (40), as well as an unpublished set of 14 strains from Mozambique; all were intact for the RDAf2 region.

From these spoligotype and deletion surveys of *M. bovis* in African nations, we concluded that strains of the Af2 clonal complex were present at high frequency in some east African countries (Uganda, Burundi, Tanzania, and Ethiopia) but were rare or absent in Algeria, Mali, Nigeria, Chad, Cameroon, Zambia, South Africa, Mozambique, and Madagascar. This observation echoes the localization of the Af1 clonal complex to west-central Africa (41) (Fig. 1).

Global distribution of Af2. In unpublished data, we have shown that a globally distributed clonal complex of *M. bovis* called European 1 (Eu1), which is defined by a specific deletion called RDEu1 and the loss of spoligotype spacer 11 (spacers 4 to 7 are usually present), is present at high frequency in many parts of the world. RDAf2 deletion analysis of a sample of 21 Eu1 strains from Great Britain (see the supplemental material) showed that they were intact at the RDAf2 locus, and reciprocal deletion analysis showed that a sample of RDAf2 strains was intact at the RDEu1 locus. We concluded that strains of the Eu1 clonal complex are not members of the Af2 clonal complex and vice versa. The phylogenetic independence of Af2 and Eu1 implies that Af2 strains are rare or absent on the British Isles, most of the New World, Australia, and New Zealand, where the Eu1 clonal complex is virtually at fixation (unpublished data).

In mainland Europe, where Eu1 is rare, we inspected previously published spoligotype surveys of cattle isolates of *M. bovis* from France, Italy, Spain, Belgium, and Portugal for isolates showing the spoligotype signature of Af2 strains: the

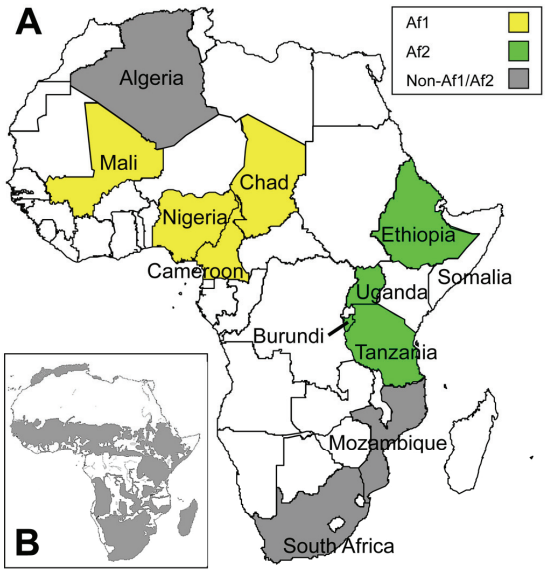


FIG. 1. Localization of the *M. bovis* Af1 and Af2 clonal complexes in Africa. (A) The four west-central African countries where Af1 strains were found to be dominant are shown in yellow, and the four east African countries where Af2 strains are highly prevalent are shown in green. Isolates of the Af1 clonal complex are very rare or not present in countries with the Af2 complex and vice versa. Countries where no Af1 or Af2 strains have been identified are labeled in gray, and countries where no isolates were studied are white. (B) Cattle distribution on the African continent (gray shaded area). (Reprinted from reference 32 with permission from the publisher.).

loss of spacers 3 to 7. We identified small numbers of isolates with the Af2 spoligotype signature from France (<2%), Italy (<2%), and Spain (<1%) (3, 10, 31, 49, 52). Sixteen (2%) of 747 *M. bovis* isolates from northern Italy had either spoligotype SB0133 (the most common Af2 spoligotype pattern in East Africa) or SB1584. RDAf2 deletion typing showed that none of the Italian strains were deleted for this region. Furthermore, in a large survey of 5,585 *M. bovis* isolates from Spanish cattle, 20 isolates were, by spoligotype pattern, possible members of the Af2 clonal complex (49). However, none of these Spanish strains were deleted for RDAf2 (see the supplemental material). We concluded that strains of the Af2 clonal complex were rare or absent in cattle outside East Africa. In this respect, Af2 again mimics the Af1 clonal complex, which has not been found in cattle outside west-central Africa (41).

Human isolates of Af2. The prevalence of bovine TB in humans is unknown in most African countries, and *M. bovis* is isolated only occasionally from humans. However, previously published studies from Uganda identified three human *M. bovis* isolates with spoligotype patterns identical to those of the commonest Af2 strains found in that country (44–45). One of these strains was deletion typed for RDAf2, and we confirmed the region to be deleted. A previously unpublished *M. bovis* strain, collected in Sweden from a patient born in Somalia, had a spoligotype identical to the commonest spoligotype found in cattle in the neighboring country Ethiopia (SB1176) and was also deleted for RDAf2, with deletion boundaries identical to those observed in strains isolated from cattle (see the supplemental material).

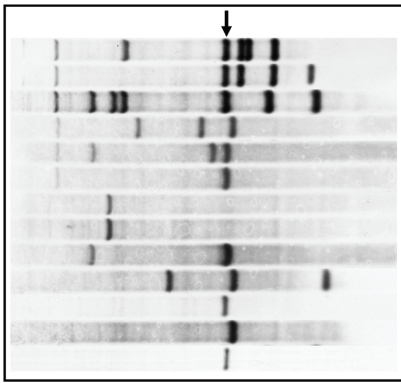
IS*6110* copy number. It has frequently been suggested that *M. bovis* isolates from cattle have only one, or a few, copies of the insertion element IS*6110* (4, 7, 15, 17–18, 50, 64). However, cattle isolates of *M. bovis* from Burundi and Uganda have been shown to have multiple copies of IS*6110* (5, 48). All 10 isolates from Burundi that we have characterized as Af2 had four or more IS*6110* copies, and five of the Af2 isolates from Uganda had six or more copies of IS*6110*. In contrast, two strains from Uganda, previously shown to have only one copy of IS*6110* (5), were found to be intact at RDAf2 and therefore were not members of the Af2 clonal complex.

To further explore the IS*6110* copy number in Af2 strains, we subjected four isolates of the Af2 clonal complex from Ethiopia to IS*6110* RFLP typing. Three of the Af2 isolates contained between four and seven IS*6110* copies, while a single Af2 strain from Ethiopia had only two copies of IS*6110* (Fig. 2). We also IS*6110* RFLP typed six strains that were intact at the RDAf2 locus (not members of the Af2 clonal complex) from Ethiopia, Mali, and Chad; four of these isolates had only one copy of IS*6110*; however, two strains, from Ethiopia and Mali, had two and three copies, respectively (Fig. 2). In total, including previously published IS*6110* RFLP data (5, 48) on strains identified here by deletion typing as Af2, 20 of 21 Af2 strains had four or more copies of IS*6110*.

DISCUSSION

We have identified an epidemiologically important clonal complex of *M. bovis* which is found at high frequency in Uganda, Burundi, Tanzania, and Ethiopia and have named this clonal complex African 2 (Af2). The Af2 clonal complex is epidemiologically important because it is commonly recovered from cattle in these four countries, but we do not yet know how phylogenetically distinct this clonal complex is from other clonal complexes of *M. bovis*. Members of the Af2 clonal complex are defined by a 14.1-kb deletion of chromosomal DNA which we have named Region of Difference Af2 (RDAf2). Sequencing of the RDAf2 region in nine isolates from the four countries has shown that the deletion boundaries are identical; in the absence of repetitive elements, or other features, flanking the RDAf2 deletion that can promote homoplastic deletions and the apparent strict clonality of *M. bovis*, we conclude that this deletion is identical by descent in strains from each of these four countries. That is, RDAf2 was deleted in the most recent common ancestor of this clonal complex, and this region is therefore deleted in all of its descendants. A definition and summary of the Af2 clonal complex are shown in Table 2.

Strains of the Af2 clonal complex can be identified by the loss of spacers 3 to 7 in the DR locus; however, this characteristic is not necessarily specific. It is theoretically possible for strains with the RDAf2 deletion to have these spacers present, although we have not yet identified such an isolate. Furthermore, because the loss of spoligotype spacers can be subject to homoplasy (54, 67), strains that are not members of the Af2 clonal complex (RDAf2 region intact) may also lack spacers 3 to 7; for example, two Nigerian strains of the African 1 clonal complex (deleted for RDAf1) (41) had also lost spacers 3 to 7,



Origin	Isolate	No of IS*6110*	RDAf2	Spoligotype
Burundi	940439	7	Deleted	SB1388
Burundi	940130	6	Deleted	SB0303
Ethiopia	BTB1356	7	Deleted	SB0133
Ethiopia	BTB0422	4	Deleted	SB1477
Ethiopia	BTB1067	4	Deleted	SB1176
Ethiopia	BTB1087	2	Deleted	SB0133
Ethiopia	BTB0915	1	Intact	SB1476
Ethiopia	BTB0893	1	Intact	SB1476
Ethiopia	BTB1192	2	Intact	SB0134
Mali	53b	3	Intact	SB1410
Mali	51b	1	Intact	SB0134
Chad	491	1	Intact	SB0944
	P3 reference			

FIG. 2. IS*6110* RFLP patterns, IS*6110* copy numbers, RDAf2 deletion types, and spoligotypes of 12 *M. bovis* isolates from Africa and of the reference *M. bovis* BCG strain P3. The arrow marks a ~1.9-kb restriction fragment commonly representing an IS*6110* copy found in the DR region of *M. bovis* strains.

as well as the signature loss of spacer 30, and were shown to be intact for RDAf2.

Af2 in Africa. We showed by reciprocal deletion analysis that strains of the Af2 clonal complex are not members of the African 1 (Af1) clonal complex, which is virtually fixed in Nigeria, Chad, and Cameroon and represents 65% of the isolates from Mali (41). Isolates from Mali that were not members of the dominant Af1 clonal complex have been given the preliminary name African 5 (Af5) based on the common loss of spacers 3 to 5 in their spoligotype pattern. RDAf2 deletion analysis of Af5 strains from Mali showed they were not members of the Af2 clonal complex, and we conclude that Af2 is rare or absent in all four of these west-central African countries.

We have also subjected strains from Algeria, South Africa, and Mozambique to RDAf2 deletion typing, and although the number of strains sampled in each of these countries was small, this supported our conclusion that the Af2 clonal complex is not uniformly distributed throughout Africa. In general, spoligotype surveys showed that strains with spacers 3 to 7 deleted are rare in these African nations, reinforcing the suggestion of localization of Af2 to cattle in East Africa (Fig. 1).

Af2 in the rest of the world. An inspection of previously published spoligotype surveys from countries throughout the world did not identify any country with more than 2% of strains with spacers 3 to 7 missing in their spoligotype patterns. In unpublished data, we have observed that another clonal complex of *M. bovis*, European 1, is virtually fixed in the British Isles, most of the New World, Australia, and New Zealand and therefore the Af2 clonal complex is rare or absent from these countries. Furthermore, large spoligotype surveys of strains from mainland Europe (10, 22, 49) and Iran (58) and RDAf2 deletion analysis of the few strains with the Af2 spoligotype signature from Italy and Spain did not identify any Af2 strains in cattle outside Africa.

We conclude that among the countries sampled, strains of the Af2 clonal complex were common in cattle only in the four east African nations; in this respect, Af2 resembles the Af1 clonal complex, which is apparently confined to cattle in west-central Africa (41). Strains of the Af2 clonal complex represent over 70% of the isolates from Uganda, Burundi, Tanzania, and Ethiopia and have not been isolated from cattle elsewhere in the world.

Localization of Af2 genotypes. If we assume that spoligotype spacers are lost and never regained, then all the Af2 spoligotype patterns described here can be derived from an ancestral spoligotype pattern equivalent to that of the vaccine strain BCG (SB0120, missing spacers 3, 9, 16, and 39 to 43), with the additional deletion of spacers 4 to 7 (SB0133). Although the number of strains sampled here from Uganda, Burundi, and Tanzania is small, the population structure of Af2 in these countries showed remarkable differences; the most common Af2 spoligotype pattern in each of the four east African countries surveyed was different. Spoligotype patterns SB0133 and SB0303 (a single spacer 13 loss derivative of spoligotype pattern SB0133) were the only Af2 patterns found in more than one country in our data set. However, the frequency of strains with these two patterns varied remarkably between countries. SB0133 was most common in our sample from Tanzania (64%) but was at much lower frequencies in the three other east African nations (from 0 to 14%), and spoligotype pattern SB0303 was common in Burundi (50%) but was only found in a single isolate of the 120 strains from Ethiopia. This observation contrasts with the spoligotype distribution of the Af1 clonal complex, for which a single ancestral-type spoligotype pattern was dominant in three of four west-central African nations (41).

To further investigate the national differences between Af2 clones, we six-locus VNTR typed a sample of eight strains with spoligotype pattern SB0133 from Tanzania and nine strains with the same spoligotype pattern from Ethiopia (Table 3). The Tanzanian strains were all of the same genotype (spoligotype plus VNTR type); however, this genotype was not found among the nine SB0133 strains from Ethiopia. Finally, a single isolate of spoligotype SB0303 from Ethiopia differed from the genotypes found in five strains with that spoligotype from Burundi (Table 3).

Both the spoligotype surveys and the genotype comparisons

TABLE 3. Countries of isolation, spoligotypes, VNTR types, and frequencies of *M. bovis* strains of the Af2 clonal complex with spoligotype patterns that were found in multiple countries

Country	Spoligotype	ETR-VNTR[a]	Frequency, no. of strains (source)
Ethiopia	SB 0133	4 2 5 4* 2 3.1	6
Ethiopia	SB 0133	3 2 5 4* 2 3.1	1
Ethiopia	SB 0133	5 2 4 3* 3 3.1	1
Ethiopia	SB 0133	5 2 4 3* 3 2.1	1
Tanzania	SB 0133	3 2 5 4* 3 3.1	8
Ethiopia	SB 0303	5 2 4 3* 3 3.1	1
Burundi	SB 0303	2 2 5 4* 3 3.1	3
Burundi	SB 0303	2 2 5 6* 3 3.1	2
Ethiopia	SB 1176	5 2 5 4* 3 3.1	6
Somalia[b]	SB 1176	5 2 5 4* 3 3.1	1 (human)

[a] Allele call for the ETR-A to -F loci (12).
[b] Strain isolated in Sweden.

suggest that the population of Af2 strains in each of these four east African countries is unique. That is, for any isolate of Af2, it should be possible, with reasonable accuracy, to determine from its genotype its country of origin. This conclusion reinforces a continuing theme of national localization of *M. bovis* genotypes initially described for Af1 strains in west-central Africa (41). However, the genotype data presented here for Af2 and, to a lesser extent, the spoligotype data must be interpreted with care. Apart from Ethiopia, the sample sizes of strains from the other three countries were very small and, more significantly, isolated in only a few localized areas. Intracountry geographical localization of *M. bovis* genotypes, as is commonly seen in the United Kingdom (54) and as was discussed in a previous study (41), could confound the observation of national localization of the Af2 genotypes presented here.

Human isolates of Af2. Support for geographical localization of Af2 genotypes comes from *M. bovis* strains isolated from humans. In Uganda, several human isolates were shown to have the same spoligotype pattern as those found in local cattle (5, 45). Furthermore, a Somali immigrant was diagnosed with abdominal TB shortly after arrival in Sweden, and the infection was confirmed as bovine TB (unpublished data). This *M. bovis* isolate was deleted for RDAf2 and had a genotype identical to those found at high frequency in Ethiopia (SB1176; 5 2 5 4* 3 3.1) (Table 3). We do not know where this patient contracted this disease, but it is possible that the original source of infection was cattle in Somalia. This epidemiological link to Somalia implies that the Af2 clonal complex may be more widely distributed across the Horn of Africa than the present study shows. However, it is possible that the Somali patient migrated via Ethiopia and was infected during transit.

Evolution of the Af2 clonal complex. The simplest explanation for the observed distribution and population structure of the Af2 clonal complex throughout these east African countries is that this clonal complex of *M. bovis* spread between these four countries. The progenitor strain, originating in one place, would have had spoligotype pattern SB0133 (spacers 3 to 7 missing) and carried the RDAf2 deletion; all Af2 spoligotype patterns described here can be generated from SB0133 by spacer loss. The country-specific population structures could have evolved by drift during the spread of the Af2 clonal complex between countries in a series of founder events or subsequently as the population expanded in each country. This explanation is similar to that used to explain the distribution of Af1 strains throughout west-central African nations (41), but with one important difference. The Af1 clonal complex of *M. bovis* was fixed in three of the four west-central African countries in which it was sampled, and therefore it was suggested that the Af1 clonal complex was transmitted through cattle naive to bovine TB. The spoligotype surveys of strains from East Africa (Uganda, Burundi, Tanzania, and Ethiopia) showed that in three of these countries non-Af2 strains of *M. bovis* are present at a reasonably high frequency (between 5 and 33%). There is no obvious relationship between the spoligotype patterns of non-Af2 strains identified in Uganda, Tanzania, and Ethiopia, and we do not know the temporal relationship between the origin of these non-Af2 strains and the Af2 clonal complex; the non-Af2 strains may have been present before the introduction of the Af2 clonal complex or may have been introduced recently from neighboring countries that have not yet been surveyed for bovine TB. This question may be resolved as more countries in Africa are surveyed for genotypes of bovine TB.

Whether the progenitor of the Af2 clonal complex evolved in Africa or evolved elsewhere and was subsequently imported to Africa is unknown, although this may be resolved when the phylogenetic relationship of this clonal complex to strains from other countries is determined by whole-genome sequencing.

The RDAf2 deletion. The large RDAf2 deletion (14.1 kb) affects 12 open reading frames on the *M. bovis* chromosome, of which 9 belong to an operon, *mce2*. *M. tuberculosis* has four homologous *mce* operons, *mce1* to *mce4* (14), whereas all *M. bovis* strains are missing the entire *mce3* operon due to a deletion, RD7, which was lost early in the lineage of animal-adapted strains that leads from the recent common ancestor with *M. tuberculosis* to *M. bovis* (55). All members of the Af2 clonal complex have, in addition, lost the *mce2* operon as a consequence of the RDAf2 deletion.

Each *mce* operon includes two *yrbE* and six *mce* genes, which show homology to ABC transporter permeases and substrate-binding proteins, respectively (13), and are believed to be involved in transport across the cell envelope. The *mce4* operon in *M. tuberculosis* has been identified as a cholesterol import system (46), while the functions of the other three *mce* operons are still unclear. However, several studies have shown that *M. tuberculosis* isolates deleted for *mce2* are attenuated in mice (1, 28, 37). It remains to be seen if strains of the *M. bovis* Af2 clonal complex, with a naturally deleted *mce2* operon, would also be attenuated in mice. However, isolates of the Af2 clonal complex collected from cattle for this study were, in general, isolated from typical tubercle lesions, suggesting that RDAf2 isolates are capable of causing typical tuberculosis-like pathology in cattle. Further work is needed to assess any differences in pathogenicity between strains of the Af2 clonal complex and those of other clonal complexes of *M. bovis*.

IS*6110* copy number. It has been suggested that *M. bovis* from cattle has only one or two copies of the transposable element IS*6110* (4, 7, 15, 17–18, 39–40, 50, 64). Most other species (or ecotypes [56]) of the *M. tuberculosis* complex have multiple copies of IS*6110*, with the exception of some *M. tuberculosis* lineages; for example, strains of the TbD1 intact or

"ancestral" lineage are frequently found to have low copy numbers of IS*6110* (20, 57). We here show that the Af2 clonal complex of *M. bovis* is, in general, multicopy (four or more) for IS*6110*. However, this is not a reliable characteristic of Af2 strains; isolate BTB1087 in this study was deleted for RDAf2 and contained only two copies of IS*6110*.

We suspect that the association between *M. bovis* and a low copy number of IS*6110* has developed because of the global distribution of an *M. bovis* clonal complex which has, in general, only one (or rarely two) copies of IS*6110* (the European 1 clonal complex; unpublished data) and is present in many developed nations with the technology to carry out IS*6110* RFLP analysis.

Strains of almost all species within the *M. tuberculosis* complex carry an IS*6110* element in the direct repeat (DR) region, which is a hot spot integration region for insertion elements (34). For *M. bovis* strains, this IS*6110* copy in the DR region is usually visualized as a ~1.9-kb PvuII restriction fragment in IS*6110* RFLP analysis (15, 19, 64). It has previously been suggested that small variations in the size of this fragment correlate with variation in the number of spoligotype spacers in the DR region that are flanking the IS*6110* DNA (64). We observed in our RFLP analysis that the single copy of IS*6110* in the two Ethiopian strains, BTB0893 and BTB0915 (both intact for RDAf2), was integrated in a much larger restriction fragment of ~4.2 kb. We suggest that this much larger fragment may be explained by the loss of the PvuII restriction site that is commonly found in spacer 36 (63); spacer 36 is absent from the spoligotype pattern of strains BTB0893 and BTB0915. Using a DR probe in RFLP analysis, we verified that a single IS*6110* copy was located in the DR region in those two strains and in the other 10 isolates examined. We concluded that all *M. bovis* strains investigated by RFLP typing in this study carried an IS*6110* copy in the DR region.

Conclusions. We have identified a second clonal complex of *M. bovis* in Africa, found at high frequency in east African cattle and with a distribution that does not overlap with the previously identified west-central African clonal complex, African 1. The geographical localization of the Af2 clonal complex to these four east African countries, and perhaps to some additional neighboring countries not yet surveyed, may have been governed by geographical features that affect cattle density, trading, and movement in this part of Africa. For example, Fig. 1B shows the cattle distribution in Africa (32), and it is interesting to note the limited links between regions of high cattle density in East Africa, where Af2 is prevalent, and regions in west-central Africa, where Af1 dominates. The uneven distribution of cattle in Africa may have contributed to localized dominance of clonal complexes of *M. bovis* in different regions of Africa.

However, these two clonal complexes, Af1 and Af2, may represent groups of strains with different selective advantages or behaviors, and comparing and contrasting the phenotypic differences between these distinct divisions within *M. bovis* may elucidate the molecular mechanisms of these differences and identify the selective forces operating on both bovine TB and its cattle host. For example, *Bos taurus* (European cattle) is common in West Africa, where the African 1 clonal complex of *M. bovis* dominates, whereas *Bos indicus* (Zebu; Asian cattle) is common in East Africa, where African 2 dominates (32–33). It will require further work to determine if the African 1 and African 2 clonal complexes are specifically adapted to these two different types of cattle or if the relationship merely represents demographic happenstance.

On a more practical level, the results presented here show that the development of simple genotype schemes for *M. bovis* within these east African countries is worthwhile and will aid eradication schemes by identifying strains imported from neighboring countries (41). Furthermore, now that the African 1 and African 2 clonal complexes have been identified, it is a simple matter to sequence chromosomes of representative isolates and gather a rich harvest of specific molecular polymorphisms to use in local epidemiological analysis. Comparative genome sequence analysis will also resolve the phylogenetic status of these clonal complexes and may show that the majority of bovine TB found in Africa originated elsewhere and has been imported to the continent relatively recently. This, in turn, will develop our understanding of the historical and phylogeographical basis of bovine TB in Africa and inform our understanding of the disease in Europe and throughout the world.

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Table 11. Spanish *Mycobacterium bovis* strains deletion typed for RDAf2. Extract from the supplementary material available at http://jb.asm.org/.

Strain ID	Spoligotype	RDAf2	Spacer 4-7	Where isolated	When isolated (Month, Year)	Species
04/0320	SB0933	intact	absent	ALBACETE	26/02/2004	cattle
04/1358	SB0933	intact	absent	JAEN	30/09/2004	cattle
04/1362	SB0933	intact	absent	JAEN	30/09/2004	cattle
97/110	SB1145	intact	absent	MADRID	16/09/1997	cattle
98/727	SB1277	intact	absent	MADRID	16/12/1998	cattle
MI06/00369	SB1145	intact	absent	AVILA	02/02/2006	cattle
MI06/06050	SB1388	intact	absent	NAVARRA	26/10/2006	cattle
MI07/00680	SB1623	intact	absent	VALLADOLID	25/01/2007	cattle
MI07/00730	SB1394	intact	absent	ZAMORA	25/01/2007	cattle
MI07/08613	SB0133	intact	absent	SALAMANCA	26/07/2007	cattle
MI07/12737	SB1145	intact	absent	AVILA	18/10/2007	cattle
MI07/13211	SB1388	intact	absent	SALAMANCA	25/10/2007	cattle
MI07/16312	SB1394	intact	absent	ZAMORA	20/12/2007	cattle
MI07/16313	SB1394	intact	absent	ZAMORA	20/12/2007	cattle
MI08/04429	SB1145	intact	absent	SALAMANCA	27/03/2008	cattle
MI09/00034	SB1623	intact	absent	CADIZ	13/01/2009	cattle
MI09/02571	SB0133	intact	absent	SALAMANCA	11/02/2009	cattle
MI09/06310	SB1388	intact	absent	SALAMANCA	27/05/2009	cattle
MI09/07395	SB1623	intact	absent	CADIZ	01/07/2009	cattle
MI09/11436	SB1623	intact	absent	CADIZ	02/12/2009	cattle

IV.3. The European 1 clonal complex of *M. bovis*

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European 1: A globally important clonal complex of *Mycobacterium bovis*

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ABSTRACT

We have identified a globally important clonal complex of *Mycobacterium bovis* by deletion analysis of over one thousand strains from over 30 countries. We initially show that over 99% of the strains of *M. bovis*, the cause of bovine tuberculosis, isolated from cattle in the Republic of Ireland and the UK are closely related and are members of a single clonal complex marked by the deletion of chromosomal region RDEu1 and we named this clonal complex European 1 (Eu1). Eu1 strains were present at less than 14% of French, Portuguese and Spanish isolates of *M. bovis* but are rare in other mainland European countries and Iran. However, strains of the Eu1 clonal complex were found at high frequency in former trading partners of the UK (USA, South Africa, New Zealand, Australia and Canada). The Americas, with the exception of Brazil, are dominated by the Eu1 clonal complex which was at high frequency in Argentina, Chile, Ecuador and Mexico as well as North America. Eu1 was rare or absent in the African countries surveyed except South Africa. A small sample of strains from Taiwan were non-Eu1 but, surprisingly, isolates from Korea and Kazakhstan were members of the Eu1 clonal complex. The simplest explanation for much of the current distribution of the Eu1 clonal complex is that it was spread in infected cattle, such as Herefords, from the UK to former trading partners, although there is evidence of secondary dispersion since. This is the first identification of a globally dispersed clonal complex *M. bovis* and indicates that much of the current global distribution of this important veterinary pathogen has resulted from relatively recent International trade in cattle.

1. Introduction

The *Mycobacterium tuberculosis* complex comprises many species and sub-species that cause tuberculosis (TB) in a variety of mammalian hosts and includes *Mycobacterium bovis*, the principle cause of tuberculosis in cattle (Smith et al., 2006a). The most notable member of the complex is *M. tuberculosis*, the most important bacterial pathogen of humans; however, the preferred host of *M. bovis* is domesticated cattle, although this pathogen can frequently be isolated from other mammals including man (Smith et al., 2006a). Because of the close genetic similarity of the *M. tuberculosis* complex of bacteria and the similarity of pathology, despite widely different host-adaptation, it has been suggested that different host-adapted forms would better be referred to as 'ecotypes' rather than species (Smith et al., 2006b). Bovine TB has been found in cattle on every continent where cattle are farmed (Amanfu, 2006; Cosivi et al., 1998).

In most of mainland Europe, the United States of America (USA), Canada, Australia, Cuba and some South American countries bovine TB has been reduced or eliminated from domestic cattle by the long term application of a test-and-slaughter policy that removed infected cattle (Amanfu, 2006; Ayele et al., 2004; Cosivi et al., 1998, 1995; Smith et al., 2006a; Thoen et al., 2006a,b). With the exception of Australia and some Caribbean Islands (Tweddle and Livingstone, 1994), many of these countries still have occasional, and sometimes persistent, outbreaks of bovine TB associated with either the import of infected cattle from other countries or the maintenance of the disease in a wildlife host.

In the United Kingdom (UK), test-and-slaughter brought the disease to a very low incidence in the 1970s. Since then the incidence of the disease has inexorably risen (Smith et al., 2006a) and the Republic of Ireland (RoI) and the UK have the highest incidence of bovine TB in the European Union (Reviriego Gordejo and Vermeersch, 2006). For the rest of the European Union bovine TB has mainly been controlled by test-and-slaughter but it continues to be a persistent problem in parts of Spain, Italy and Portugal (Pavlik, 2006).

Bovine TB has been shown to be present in most countries in Africa but in general, due to economic constraints, the true extent of the disease has not been evaluated (Ayele et al., 2004; Cosivi et al., 1995). The exception is South Africa where an extensive test-and-slaughter has reduced the disease in cattle to minimal levels (Michel et al., 2008). In North America bovine TB is endemic in Mexican cattle but has been largely eliminated from cattle in the USA and Canada (Milian-Suazo et al., 2008; Wobeser, 2009). However, in the USA a persistent problem has been reported in white-tailed deer in Michigan, as well as small breakdowns in Minnesota and Molokai Island, Hawaii (associated with feral swine) (Bany and Freier, 2000). The USA also suffers from the occasional import of bovine TB in Mexican cattle (Milian-Suazo et al., 2008; Rodwell et al., 2010). In Canada there are two areas where wildlife populations are still infected with bovine TB; free-ranging populations of wood bison in and around Wood Buffalo National Park, which straddles the provinces of Alberta and the Northwest Territories, and deer and elk (wapiti) in Riding Mountain National Park, Manitoba (Wobeser, 2009). The Bovine Tuberculosis Eradication Program in Mexico has successfully reduced the prevalence of TB in cattle in certain regions. Beef cattle in the northern-most states have the lowest prevalence, averaging between 0.01 and 0.25% (Ritacco et al., 2006). However, the prevalence of *M. bovis* in Mexican dairy cattle is higher, with an estimated infection rate in this population of 16–17% (Milian-Suazo et al., 2000; Milian et al., 2000).

Bovine TB is endemic in cattle in South America (de Kantor et al., 2008). About 70% of the cattle are found in areas with high disease

prevalence although nearly 17% are in areas virtually free from TB (de Kantor and Ritacco, 2006). For the rest of the world, bovine TB is thought to be endemic in cattle and there have been, with notable exceptions, few molecular epidemiological surveys of the strains present in each country (Cosivi et al., 1998; Jeon et al., 2008; Tadayon et al., 2006; Thoen et al., 2006a).

In the North American hotspots bovine TB persistence is associated with maintenance in an alternative wildlife host (white-tailed deer in Michigan, pigs in Hawaii and buffalo in the Canadian National Parks (Rhyan and Spraker, 2010)). In a similar manner the persistence of bovine TB in New Zealand cattle is associated with brush-tailed possums (Tweddle and Livingstone, 1994) and the failure of the test-and-slaughter in the UK is associated with disease maintenance in Eurasian badgers (*Meles meles*) (Gallagher and Clifton-Hadley, 2000; Jenkins et al., 2010). In South Africa, bovine TB is thought to have been transmitted from cattle to buffalo in both the Kruger National Park and Hluhluwe-iMfolozi Park and is affecting several wildlife species in these national parks (Michel et al., 2009). It is becoming a feature of bovine TB control, internationally, that the test-and-slaughter protocol for cattle, that worked so well in mainland Europe, the USA and Australia, is failing in other countries because of a wildlife maintenance host for the disease (Van Campen and Rhyan, 2010).

Spoligotyping, a PCR and hybridisation technique, is a common molecular typing method applied to isolates of the *M. tuberculosis* complex and identifies polymorphism in the presence of spacer units in the direct repeat (DR) region (Kamerbeek et al., 1997; van der Zanden et al., 1998). The DR region is composed of multiple, virtually identical, 36-bp repeats interspersed with unique DNA spacer sequences of a similar size (direct variant repeat or DVR units). The DR region may contain over 60 DVR units, however, 43 of the spacer units were initially selected and are used in the standard spoligotyping method used to type strains of the *M. tuberculosis* complex (Groenen et al., 1993; Kamerbeek et al., 1997; van Embden et al., 2000). The DR region is polymorphic because of the loss (deletion) of single or multiple spacers, and each spoligotype pattern from strains of the animal-adapted lineage of the *M. tuberculosis* complex is given a unique identifier by www.Mbovis.org.

The population structure of the *M. tuberculosis* complex of bacteria is apparently highly clonal and no cases of transfer and recombination of house-keeping genes between strains have been identified (Cole et al., 1998; Gutacker et al., 2002; Hershberg et al., 2009; Smith et al., 2003, 2006a). However, there have been reports of between-strain recombination in close proximity to the hypervariable and immunogenic *PE* and *PPE* genes (McEvoy et al., 2009) In a strictly clonal population the loss by deletion of unique chromosomal DNA cannot be repaired by recombination from another strain and the deleted region will act as a molecular marker for the strain and all its descendants. Deletions of specific chromosomal regions (Regions of Difference – RDs or Large Sequence Polymorphisms - LSPs) have been very successful at identifying phylogenetic relationships in the *M. tuberculosis* complex (Brosch et al., 2002; Gagneux et al., 2006; Gagneux and Small, 2007; Huard et al., 2006; Mostowy et al., 2005; Narayanan et al., 2008; Smith et al., 2006a,b; Tsolaki et al., 2005). Deletions of spoligotype spacers generate novel spoligotype patterns, however, the loss of spacers is so frequent that identical spoligotype patterns can occur independently in unrelated lineages (homoplasy) and therefore a spoligotype pattern may be an unreliable indicator of phylogenetic relationship (Schurch et al., 2011; Smith et al., 2006a; Warren et al., 2002).

In previous work two other epidemiologically important clonal complexes of *M. bovis*, named African 1 (Af1, dominant in Cameroon, Nigeria, Chad and Mali) and African 2 (Af2, at high frequency in East Africa) have been identified (Berg et al., 2011; Muller et al., 2009). All members of the Af1 clonal complex of *M. bovis* are defined by a specific chromosomal deletion (RDAf1) and lacked spacer 30 in their spoligotype pattern and strains of the Af2 clonal complex are identified by a specific deletion (RDAf2) and are associated with the absence of spoligotype spacers 3 to 7. Here, we show that a third clonal complex, called European 1 (Eu1), is dominant in the Republic of Ireland and the UK, some former British colonies, Korea and the New World (with the exception of Brazil). This is the first identification of a globally important clonal complex of *M. bovis* that has, apparently, been spread throughout the world by the International trade in cattle.

2. Materials and methods

2.1. Bacterial strains, spoligotyping and sequencing

Details of all strains deletion typed for this manuscript are given in the supplementary data. Strains were spoligotyped according to the method of Kamerbeek et al. (1997) with minor modifications (Cadmus et al., 2006). Sequencing across the deletion boundary of RDEu1 was carried out using standard sequencing methods using the RDEu1 deletion primers.

2.2. RDEu1 deletion typing

The status of the RDEu1 region was assessed by a PCR assay using a pair of primers located at a suitable distance flanking the deletion boundary (RDEu1 primer set A). The forward primer was RDEu1_FW (5′-CCGATGAACTTGGCCCACAG-3′ (position 1767904 to1767923 in H37Rv) and the reverse primer was RDEu1_ Rv(5′–CGTGGTGGTGGGATGTCTTG-3′ (position 1769110 to 1769091 in H37Rv). Final PCR reactions contained 2 μl of heat-killed mycobacterial cell supernatant, 10 μM HotStartTaq Master Mix (Qiagen), 1 μM of primers RDEu1_FW and RDEu1_Rv, and sterile distilled water to a final volume of 20 μl. Thermal cycling was performed with an initial denaturation step of 15 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 58 °C and 2.5 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. PCR products were visualised after electrophoresis on a 1% agarose gel. A 1206 bp fragment was generated if the RDEu1 region was intact and a 400 bp fragment if the region was deleted. Strains CHAD491 (RDEu1 intact) and AF61/2122/97 (RDEu1 deleted) were used as controls.

2.3. Measuring the frequency of Eu1 strains

To determine the maximum frequency of Eu1 clonal complex strains in a population the following algorithm was used. From previously published spoligotype surveys for a country isolates of the most common spoligotype patterns, usually several of each, were deletion typed. We also surveyed as many minor clones with spacer 11 missing as possible. Assuming that spoligotype patterns marked clones this deletion analysis was used to determine, from the spoligotype survey, the basic frequency of Eu1 clonal complex strains in a population. To determine the maximum possible frequency of Eu1 clonal complex strains in the population we then added to this basic frequency the frequency of all strains in the spoligotype population surveys that had spacer 11 missing for which RDEu1 deletion results were unavailable.

3. Results

3.1. Strains with spacer 11 absent

It has previously been shown that many strains of *M. bovis* isolated from cattle in the RoI and the UK have a spoligotype pattern lacking spacer 11 (Smith et al., 2006a). Spacer 11 is missing

in over 96% of the 55,000 spoligotyped isolates of *M. bovis* found in Great Britain (GB) [Veterinary Laboratories Agency (VLA), Weybridge, UK, Spoligotype Database, 1994–2009] and is missing in all 16,373 isolates of *M. bovis* from Northern Ireland (NI) (Agri-Food and Biosciences Institute (AFBI), Belfast, UK, Spoligotype Database, 2003–2009]. Furthermore, in an analysis of 452 *M. bovis* isolates from both cattle and other animals in the RoI the total of twenty spoligotype patterns identified were also deleted for spacer 11 (Costello et al., 1999). In total, spacer 11 was missing from the spoligotype pattern of 99% of the *M. bovis* isolates from RoI and UK.

3.2. Identification of a specific deletion—RDEu1

A deletion, RD17 and here called RDEu1, has previously been shown to be phylogenetically informative among strains of *M. bovis* (Gordon et al., 2001). We examined the regions surrounding this deletion and determined that they show no similarity to insertion sequences or repetitive DNA, that there are no direct or inverted repeats in the regions immediately flanking the deletion and that they show the same %GC content as the rest of the *M. bovis* genome. These observations suggest that this region is not prone to independently generating deletions and that deletion RDEu1 may provide a suitable phylogenetic marker for a clonal complex of *M. bovis*.

In an unpublished analysis of approximately 500 randomly selected strains isolated from cattle in each of the three regions (GB, NI and RoI) – we identified the spoligotype patterns that were unique to each region, giving a total of 53 spoligotype patterns. We determined the frequency of the RDEu1 deletion among a sample of strains from this population survey using a simple PCR deletion assay. An isolate of every available spoligotype pattern was assayed for the presence of the RDEu1 deletion (RoI 25 strains, NI 11 strains, GB 13 strains). For these 49 strains RDEu1 was deleted in all but the strain with spoligotype SB0134 (spacer 11 present) from GB.

The RDEu1 region was deleted in a further 130 strains from GB, 240 strains from NI and 90 strains from RoI chosen to represent the spoligotype diversity in each region. Only strains with spoligotype pattern SB0134 (spacer 11 present), were intact at the RDEu1 region ($n = 10$). We conclude that a clonal complex of *M. bovis* characterised by the deletion of region RDEu1 was ubiquitous in the RoI and UK. This clonal complex is marked by the loss of spoligotype spacer 11. However, the most common spoligotype pattern associated with this clonal complex was SB0140 which has spacer 6 absent as well as spacers 8 to 12, in addition to spacers 3, 16, and 39–43, which are absent in all *M. bovis* (Smith et al., 2006b) strains. We named this clonal complex of *M. bovis* European 1 (Eu1).

3.3. Eu1 in mainland Europe

To determine the frequency of the Eu1 clonal complex in mainland Europe we have used previously published large surveys of strains of *M. bovis* from countries to identify the common spoligotypes present in the population. A sample of strains, representing the most common spoligotype patterns, were then analysed for the status of the RDEu1 region. From this analysis, and assuming that the spoligotype pattern marks a clone, we determined the maximum percentage of strains in each population that could represent the Eu1 clonal complex. That is, we assumed all strains with spacer 11 missing were potential members of the Eu1 clonal complex and then used the PCR deletion assay to eliminate some spoligotypes and to test the linkage between the loss of spacer 11 and the deletion of RDEu1.

The results of analysing the population structure of *M. bovis* for the presence of the Eu1 clonal complex in population size samples from Spain, Portugal, Italy, Belgium and France are shown in Table 1 and Fig. 1. The most common spoligotype pattern of the Eu1 clonal complex was SB0140 (see above) which was found in every country except Belgium (Allix et al., 2006). Here, and for all other strains analysed in this study, strains with region RDEu1 deleted also had spoligotype spacer 11 deleted. Details of all strains deletion typed in this study can be found in the supplementary data.

To gain insight into the population structure of *M. bovis* in The Netherlands prior to the eradication of bovine TB in cattle in 1990 we have analysed a small set of isolates from elderly Dutch patients (all born prior to 1933). No strains of the Eu1 clonal complex were identified by deletion typing and, assuming that these patients were infected with *M. bovis* prior to the eradication of the disease in cattle, these data suggest that the Eu1 clonal complex may have been rare in The Netherlands (Table 1).

In a similar manner we analysed 20 isolates from humans born in Sweden before 1940; bovine TB was eradicated from cattle in Sweden in 1958 (Szewzyk et al., 1995). The majority of these human isolates had spoligotype patterns identical, or similar to the spoligotype pattern of vaccine strain BCG (SB0120, $n = 16$, spacer 11 present and RDEu1 intact), however, five strains with spoligotype pattern SB0130 were deleted for spacer 11 and RDEu1 (Table 1). Because we do not know where these patients were infected with bovine TB we can only conclude that the Eu1 clonal complex was probably at much lower frequency in Sweden, prior to its eradication from cattle, than it currently is the RoI and UK.

From Germany 39 isolates of *M. bovis* representing the most common *M. bovis* spoligotype patterns found in 166 patients

Table 1
The frequency of the Eu1 clonal complex of *M. bovis* in the European nations surveyed by deletion typing and spoligotyping.

Country	Reference	RDEu1 deletion surveys of European strains				
		Number of isolates	Number of spoligotype patterns	Percentage of strains with spacer 11 missing	Number of strains deletion typed for RDEu1	Maximum % of Eu1 strains[a]
Great Britain	This study	490	13	97.3	13	97.3
Northern Ireland	This study	528	11	100.0	11	100.0
Republic of Ireland	This study	503	29	100.0	25	100.0
France	Haddad et al. (2001)	1349	153	14.2	95	13.8
Portugal	Duarte et al. (2008)	283	29	11.8	58	7.6
Spain	Rodriguez et al. (2009)	6215	252	10.7	45	6.1
Italy	Boniotti et al. (2009)	1503	76	1.0	40	<1.0
Belgium	Allix et al. (2006)	127	17	49.6	8	<1.0
The Netherlands	This study	41 (humans)	18	17.0	15	0.0
Sweden	This study	20 (humans)	5	25	20	25.0
Germany	Kubica et al. (2003)	176 (human, animal)	59	36.9	44	<1.0

[a] The percentage of strains with spacer 11 missing was taken as the starting point 1 for calculating the maximum 2 percentage of the Eu1 clonal complex in each population. Then strains with the commonest spoligotype patterns were 3 assayed for the RDEu1 deletion. Strain with spacer 11 deleted that were not members of the Eu1 clonal complex reduced 4 the maximum possible percentage of the Eu1 clonal complex in each population.

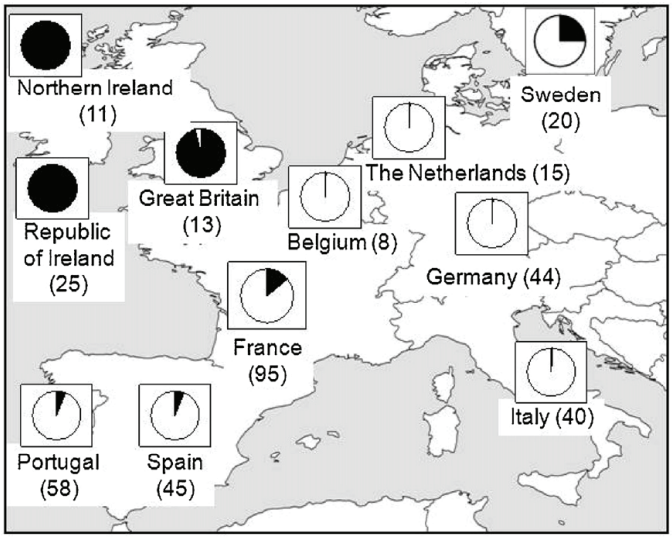

Fig. 1. Distribution of the Eu1 clonal complex of *M. bovis* throughout Europe. The pie charts show the proportion of isolates that are members of the Eu1 clonal complex; black = Eu1, white = other clonal complexes. The number of strains deletion typed for RDEu1 in each region are shown in parentheses. Strains of Eu1 are dominant in the RoI and the UK, at less than 14% frequency in France and the Iberian Peninsula and rare in the other countries surveyed. The proportion of Eu1 strains in Sweden, The Netherlands and Germany was determined from human samples assuming they reflect the population structure of bovine TB prior to its elimination from cattle.

diagnosed with TB not caused by *M. tuberculosis* between 1999 and 2001 (Kubica et al., 2003) were tested by RDEu1 deletion analysis. We also tested five strains of *M. bovis* that were isolated from animals during the same period. All but one strain, isolated from a human born in the USA, were intact for RDEu1. These data suggest, assuming that these isolates represent a sample of the bovine TB population present in German cattle prior to its elimination in 1997 (Hartung, 2001), that the RDEu1 clonal complex was rare in Germany.

3.4. Eu1 in Africa

It has previously been shown that the African 1 clonal complex of *M. bovis*, defined by deletion RDAf1 and marked by the loss of spacer 30, is dominant in Nigeria, Chad, Cameroon and Mali (Muller et al., 2009). The RDEu1 region was intact in a group of Af1 strains from these countries ($n = 26$) showing that the Af1 clonal complex and the Eu1 clonal complex are phylogenetically distinct clonal complexes. In a reciprocal experiment, RDAf1 was intact in a collection of Eu1 strains from GB representing the local spoligotype diversity ($n = 21$, unpublished data) confirming that Eu1 and Af1 are phylogenetically distinct. Because of the previously documented dominance of Af1 in Nigeria, Chad and Cameroon (over 90% of strains) we can conclude that the Eu1 clonal complex was absent or at low frequency in these three West-central African countries. In Mali 65% of the isolates are Af1 (Muller et al., 2009) and the presence of the RDEu1 region in the most common non-Af1 strain from Mali (SB0134) suggests that the Eu1 clonal complex is also rare or absent in Mali (Table 2).

Another clonal complex of *M. bovis*, marked by both a deletion (RDAf2) and a specific spoligotype signature, is present at high

Table 2
The frequency of the *M. bovis* Eu1 clonal complex in the African nations surveyed by both deletion typing and previously published spoligotype surveys.

Country	Reference	Surveys of African strains					
		Type of survey	Number of strains	Number of spoligotype patterns	Percentage of strains with spacer 11 missing	Number of strains deletion typed for RDEu1	Maximum % of Eu1 strains
Nigeria	Muller et al. (2009)	Abattoir	178	34	4.5	3	0.0
Cameroon	Muller et al. (2009)	National	75	10	13.0	16	0.0
Mali	Muller et al. (2009)	Abattoir	20	7	0.0	3	0.0
Chad	Muller et al. (2009)	Abattoir	65	13	1.5	5	0.0
Ethiopia	Berg et al. (2009)	National	58	7	0.0	15	0.0
Burundi	Rigouts et al. (1996)	National	10	3	0.0	10	0.0
Tanzania	Muller et al. (2009)	Abattoir	14	3	36.0	13	7.0
Mozambique	Unpublished	Localised	12	1	0.0	12	0.0
South Africa	Michel et al. (2008)	National	50	12	62.0	35	62.0
Algeria	Sahraoui et al. (2009)	Abattoir	88	22	1.0	Spoligotype survey[a]	1.0
Uganda	Oloya et al. (2007)	Humans	19	10	0.0	Spoligotype survey	0.0
Uganda	Asiimwe et al. (2009)	Abattoir	11	6	0.0	Spoligotype survey	0.0
Madagascar	Rasolofo Razanamparany et al. (2006)	National	100	12	2.0	Spoligotype survey	2.0
Zambia	Munyeme et al. (2009)	Localised	31	10	6.4	Spoligotype survey	6.4

[a] Previously published surveys by spoligotype only.

frequency in Uganda, Ethiopia, Burundi and Tanzania (Berg et al., 2009, 2011). This East African clonal complex of *M. bovis* has been designated African 2 (Af2) and represents over 70% of all cattle isolates from each of these East African countries. Strains of the Af2 clonal complex are intact at the RDEu1 region and spoligotype surveys of these countries showed only very low levels of strains with spacer 11 missing (Berg et al., 2011). We surveyed a sample of available strains ($n = 38$) from Ethiopia, Burundi and Tanzania for the Eu1 specific deletion including 27 strains of the Af2 clonal complex; no strains deleted for RDEu1 were identified.

A set of twelve strains of *M. bovis* from the Buzi District of Central Mozambique all had spoligotype pattern SB0961 (spacer 11 present) and were shown to be intact at RDEu1. Previously published surveys of *M. bovis* strains from Madagascar, Zambia and Algeria also suggest that spoligotype patterns with spacer 11 missing are rare in these countries (Munyeme et al., 2009; Rasolofo Razanamparany et al., 2006; Sahraoui et al., 2009). We concluded that in the African countries surveyed the Eu1 clonal complex was absent or at very low frequency (Table 2).

3.5. Eu1 in southern Africa

We analysed 35 strains isolated from South African cattle between 1991 and 2008. Many isolates from cattle in South Africa had spoligotype patterns similar to those found in the RoI and UK (Michel et al., 2008). All but eight strains were deleted at the RDEu1 region and we concluded that the Eu1 clonal complex was common in cattle in South Africa. A single isolate from Swaziland was also deleted for RDEu1.

The molecular epidemiology of *M. bovis* isolates from free ranging wildlife in South African game reserves, Kruger National Park (KNP) and Hluhluwe-iMfolozi Park, KwaZulu-Natal (HiP), has been described (Michel et al., 2009). Strains from KNP were characterised by the loss of spacer 21 (SB0121) whereas strains from HiP were characterised by the loss of spacer 11 (SB0130). Twelve strains isolated from various animals from the KNP were intact for RDEu1, however, eight strains of spoligotype pattern SB0130 isolated from buffalo in the HiP were all deleted for RDEu1 and therefore members of the Eu1 clonal complex.

3.6. Eu1 in South America

We tested 77 isolates from Argentina, mainly from cattle, and 43 isolates from swine for the presence of the RDEu1 deletion; all but eight strains were deleted for both spacer 11 and RDEu1. We also analysed a collection of 30 strains from cattle isolated throughout Chile. The commonest spoligotype pattern among Chilean isolates was SB0140 and all but one of the isolates were deleted for RDEu1 and lacked spacer 11. Ten strains from the most important dairy region in Ecuador, all with spoligotype pattern SB0980 (a single spacer loss derivative of SB0140), were analysed; all strains were deleted for RDEu1 and lacked spacer 11. Finally, a collection of strains from Brazilian cattle ($n = 29$) and goats ($n = 7$) were deletion assayed for RDEu1. In contrast to the results for other South American countries only six of the 36 strains (all from cattle) were deleted for RDEu1.

3.7. Eu1 in North America, Australia and New Zealand

A previously reported spoligotype survey of 84 Mexican and American *M. bovis* isolates from cattle, deer, and feral pigs grouped the strains into 27 clusters named A to AA (Milian-Suazo et al., 2008). Strains with spacer 11 present were only found in clusters V, W, X and Y. Thirty-eight isolates representing the commonest clusters identified from both Mexico and the USA were assayed for the status of the RDEu1 region by deletion typing. All strains, except single isolates representative of clusters V, W, X and Y, were deleted for RDEu1. From Canada, a sample of strains ($n = 10$) from the Riding Mountain Eradication Area, mainly from elk (Lutze-Wallace et al., 2005), were analysed for the RDEu1 deletion. All strains were deleted for the RDEu1 region.

We concluded that the Eu1 clonal complex was common in the USA and Mexico as well as Riding Mountain National Park in Canada. Both the Michigan strains, associated with white-tailed deer, and the Hawaiian strains associated with feral pigs were also members of the Eu1 clonal complex.

We deletion surveyed 34 strains from Australia, mainly isolated prior to 1994; all were deleted for the RDEu1 region and therefore members of the Eu1 clonal complex. Sixteen strains from New Zealand, isolated from cattle between 1989 and 2003, were deletion typed for RDEu1; all 16 were deleted for RDEu1. We concluded that both Australia and New Zealand were dominated by strains of the Eu1 clonal complex.

3.8. Eu1 in Asia

We analysed 56 *M. bovis* strains isolated from dairy cattle throughout the Gyeonggi-do province of Korea (Jeon et al., 2008); 75% of the strains were of spoligotype SB0140 and all 56 isolates were deleted for RDEu1. We also RDEu1 deletion typed two strains of *M. bovis* isolated from Taiwanese nationals (SB0265, spacer 11 present) representing the two major VNTR types of this spoligotype found in Taiwan (Jou et al., 2008). Both these human isolates were intact at RDEu1. A single, previously unpublished isolate from a human with spoligotype pattern SB1040 (spacer 11 missing) was deleted for RDEu1. Furthermore, no RDEu1 deleted strains were found in a survey of 20 animal isolates suggesting that the Eu1 clonal complex is rare or absent in Taiwan (data not shown).

Spoligotype surveys of *M. bovis* isolates from TB-test reactor cattle in 24 of the 28 Iranian provinces where bovine TB has been reported showed either BCG-like spoligotype patterns (SB0120, spacer 11 present, 41% of isolates) or simple variants of this ancestral pattern (Tadayon et al., 2008). We selected a sample of 47 strains from these surveys for deletion analysis and, as expected, all strains were intact at RDEu1.

In 2006 eight strains of *M. bovis* with an unusual combination of phenotypic and biochemical characteristics were isolated from humans from the oblast of Kostanajskaya in north Kazakhstan (Kubica et al., 2006). Seven of these strains, with spoligotype pattern SB0131, a single spacer loss derivative of Eu1 type SB0130, were deleted for RDEu1.

3.9. Reference strains of M. bovis

The neotype strain of *M. bovis*, NCTC 10772 (ATCC 19210), was obtained from the National Collection of Type Cultures and spoligotyped as SB0267 (spacer 11 missing) and was deleted for RDEu1. This strain was isolated by W.D. Yoder in Texas from a granulomatous lesion in a lymph node of a 6-month-old heifer in 1965. The strain AN5 that is used worldwide for bovine PPD production was originally isolated in England around 1948 (Paterson, 1948) and has spoligotype pattern SB0268 (missing spacer 11) and is deleted for RDEu1. The *M. bovis* progenitor of the vaccine strain, BCG, was isolated by Nocard in France in 1902 from a cow with tuberculous mastitis. While this *M. bovis* strain was lost, we can infer its spoligotype from the BCG derivative, which has spoligotype pattern SB0120 (spacer 11 present). However, recently a BCG strain with a noncanonical spoligotyping profile has been identified (Mokrousov et al., 2010). Strains BCG Sweden, Danish, Russia, Tice, Frappier and Tokyo (Garcia Pelayo et al., 2009) are intact for RDEu1. Strain ATCC35723 was originally isolated from a

cow by A.G. Karlson at the Mayo Clinic, Rochester, Minnesota and he deposited it in the Trudeau Mycobacterial Collection in 1950 where it was designated TMC405. This strain has spoligotype pattern SB1185 (spacer 11 missing) and is deleted for RDEu1. Strains NCTC8438 and NCTC9320 were isolated from English cows in 1945 and 1954, respectively; both strains are of spoligotype SB0140 and deleted for RDEu1. *M. bovis* strain AF 61/2122/97, the first *M. bovis* strain to have its entire genome sequenced (Garnier et al., 2003), was isolated from an English cow in 1997, has spoligotype pattern SB0140 and is deleted for RDEu1.

To confirm that the RDEu1 deletion was identical by descent we nucleotide sequenced across the deletion boundary in a total of 89 isolates from 10 countries. The RDEu1 deletion boundary was identical in all 89 isolates.

4. Discussion

We have identified a globally important clonal complex of *M. bovis* by a deletion analysis of 1014 strains from over 30 countries and have named this clonal complex European 1 (Eu1). Members of this clonal complex are defined by a previously identified 806 bp deletion (RD17) of chromosomal DNA which we have named Region of Difference Eu1 (RDEu1) (Gordon et al., 2001). Sequencing across the RDEu1 deletion boundaries in many isolates has shown that the deletion boundaries are identical and, in the absence of repetitive elements flanking RDEu1 or other features promoting deletions, and the apparent strict clonality of *M. bovis* (Smith et al., 2006a), we conclude that this deletion is identical by descent in these strains throughout the world. That is, RDEu1 was deleted from the most recent common ancestor of this clonal complex and this region is therefore absent in all descendants of that most recent common ancestor. A definition and summary of the Eu1 clonal complex is shown in Table 3.

Strains of the Eu1 clonal complex can be identified by the loss of spacer 11 in the spoligotype pattern although this characteristic is not necessarily specific for this clonal complex. Because the loss of spacers in spoligotype patterns can be homoplastic (Smith et al., 2006a; Warren et al., 2002), strains that are not members of the Eu1 clonal complex (RDEu1 region intact) can also lack spacer 11; for example the strains with spoligotype pattern SB1284 from Spain (supplementary data). Furthermore, it is theoretically possible that the most recent common ancestor of the Eu1 clonal complex had RDEu1 deleted and had spacer 11 present; the loss of spacer 11 could have happened later and then become the major sub-clone of the Eu1 clonal complex. However, although all 476 strains that were shown to be deleted for RDEu1 in this study were also deleted for spacer 11 (supplementary data). The spoligotype signature of the Eu1 clonal complex, as well as the spoligotype signatures of the Af1 and Af2 clonal complexes, should be used as a guide to direct deletion analysis. It is the deletions that define membership of these clonal complexes and not spoligotype signature.

The Eu1 clonal complex showed a remarkable difference in frequency throughout Western Europe (Table 1). Strains of this clonal complex were virtually fixed in the RoI and UK (99%), were at less than 14% in the Iberian Peninsula and France and rare or absent in Belgium and Italy. If surveys of strains from elderly human patients can be used to indicate the population structure of bovine TB prior to its elimination from cattle then our data suggest that the Eu1 clonal complex was rare in Germany, Sweden and The Netherlands prior to its eradication from cattle (Fig. 1). In more eastern European countries spoligotype surveys suggest that strains of the Eu1 clonal complex are at low frequency and strains of *M. caprae* are more common (Pavlik, 2006).

Throughout most of the African countries surveyed and Iran, the Eu1 clonal complex is apparently at low frequency, with the exception of South Africa, where Eu1 strains represent just over 60% of strains isolated from cattle. We show that Eu1 strains are common in wildlife in the Hluhluwe-iMfolozi Park, while another clonal complex has been established in Kruger National Park.

However, the Eu1 clonal complex dominates most of the South American countries assayed with the exception of Brazil.

Strains from Brazil had spoligotype patterns similar to the vaccine strain BCG (SB0120) or were lacking spacer 21 and the difference in the population structure of *M. bovis* in Brazil, compared to neighboring South American countries is supported by further spoligotype analyses from that country (Viana-Niero et al., 2006; Zanini et al., 2005; Zumarraga et al., 1999). Although there is an obvious historical difference between Portuguese speaking Brazil and the rest of Spanish speaking South America the current populations of *M. bovis* in Spain and Portugal do not reflect the population structure differences between Brazil and the rest of South America (Eu1). Both Spain and Portugal have similar population structures for *M. bovis*; strains missing spoligotype spacer 21 are common, the Eu1 clonal complex is at low frequency (6%) and the BCG-like spoligotype pattern (SB0120) is rare (Boniotti et al., 2009; Duarte et al., 2008; Rodriguez et al., 2009).

In the USA the Eu1 clonal complex is common and included RDEu1 deleted strains isolated from coyotes, cattle and white-tailed deer in Michigan and from feral pigs in Hawaii. The strains identified as members of the Eu1 clonal complex in Michigan, Hawaii and New Mexico are distinct in lacking spacers 5 to 13 (SB1165) and similar spoligotypes are found in Mexico where Eu1 is also common (Milian-Suazo et al., 2008). Strains from the southern states of the USA are, in general, more similar to SB0140, lacking spacer 8 to 12 and strains of this type are common in Mexico (Cobos-Marin et al., 2005; Cousins and Roberts, 2001; Milian-Suazo et al., 2008).

Our Canadian sample of Eu1 strains was isolated from deer (elk) at the Riding Mountain National Park (RMNP) however this may not reflect the bovine TB that was present in Canadian cattle prior to its general elimination from cattle in 2005 (Wobeser, 2009). The origin of bovine TB in the RMNP may have involved introduced bison whose ultimate origin was the USA (Wobeser, 2009).

Table 3
Definition and summary of the European 1 (Eu1) clonal complex of *M. bovis*.

European 1 (Eu1) clonal complex of *M. bovis*	
Definition	Presence of deletion RDEu1 (806 bp in *TreY*)
Spoligotype marker	Absence of spacer 11
Spoligotype signature[a]	11011111010111101111111111111111111111100000 (SB0130)
Most common	11011010000011101111111111111111111111100000 (SB0140)
Distribution	At high frequency in: The British isles, South Africa, Australia, New Zealand, The New World (except Brazil), Korea At low frequency (<10%) in: Brazil, Spain, Portugal found in many other countries
IS6110 copy number	1 or 2 copies (infrequently 3 or 4)

[a] The spoligotype signature represents the assumed spoligotype pattern in the progenitor strain of this clonal complex and is shown as a series of 1s and 0s with 1 representing hybridisation to the spacer and 0 representing absence of hybridisation. International names for these spoligotype patterns were assigned by www.Mbovis.org.

However, the spoligotype patterns of strains from the RMNP are distinctly different from strains currently found in the USA.

The RDEu1 region was deleted in all strains from Australia and New Zealand and the fixation of the Eu1 clonal complex in Australia prior to its elimination in 1997 is supported by the previously recorded absence of spacer 11 in the spoligotype pattern of 211 Australian *M. bovis* isolates surveyed in 1998; the most common pattern in this survey was SB0140 (72%) (Cousins et al., 1998). However, the populations of *M. bovis* in these two English speaking nations are not identical. The small survey of New Zealand strains presented here suggests that strains with spoligotype pattern SB0130, the presumptive ancestral spoligotype pattern of the Eu1 clonal complex (Table 3), are more common in New Zealand (9 of 16 strains) than Australia [not seen in a spoligotype survey of 211 strains (Cousins et al., 1998)]. It has been pointed out before that New Zealand is an isolated island nation and possibly only a limited group of *M. bovis* was introduced (Collins et al., 1993).

In most of Asia, both the population structure and prevalence of bovine TB is unknown, however, this study gives a first indication to where the Eu1 clonal complex is distributed. We did not identify any strains of the Eu1 clonal complex in Iran, however, in the Republic of Korea, where bovine TB affects more than 500 dairy cattle each year and causes major economic losses in spite of a continued test-and-slaughter (Jeon et al., 2008; Wee et al., 2009), isolates from dairy cattle in Gyeonggi-do province of the Republic of Korea (Jeon et al., 2008) were deleted for Eu1 and the spoligotype patterns were of two main types SB0140 (over 75%) or SB1040, a spacer deletion derivative of SB0140. Finally, Eu1 strains were identified in humans from a rural area of northern Kazakhstan (Kubica et al., 2006).

4.1. The RDEu1 deletion

The RDEu1 deletion is 806 bp long and is located entirely within the gene for malto-oligosyltrehalose synthase (*treY*) which encodes an enzyme in the biosynthesis of the disaccharide trehalose (De Smet et al., 2000). The deletion truncates the protein and causes a frameshift which presumably affects the catalytic function of the enzyme. Three biosynthetic pathways for the production of trehalose have been identified in bacteria (Kaasen et al., 1992; Maruta et al., 1996; Tsusaki et al., 1997) and screening of the *M. tuberculosis* genome shows that homologs of all three biosynthetic pathways are present (De Smet et al., 2000). Furthermore, cell-free extracts from *M. bovis* BCG, which is intact at RDEu1, were also observed to catalyze the production of trehalose from a variety of substrates (De Smet et al., 2000) suggesting that the ancestral *M. bovis* strain (RDEu1 intact) could synthesise trehalose via each of these three biosynthetic pathways. The existence of multiple biosynthetic pathways and the resulting redundancy in trehalose synthesis, suggests that the loss of one pathway, caused by deletion RDEu1, may be selectively neutral.

4.2. Diaspora from the UK?

The presence of the Eu1 clonal complex of *M. bovis* in so many trading partners and English speaking former colonies of the UK (Fig. 2) does offer a simple explanation for the global distribution of this clonal complex (Cataldi et al., 2002). The suggestion that the UK was the epicenter for the distribution of the Eu1 clonal complex can be supported by the large number of modern cattle types that were originally bred there (Decker et al., 2009). For example, Hereford beef cattle, bred in Herefordshire, UK in the 18th century, have since been exported and re-exported to become the most numerous and widely distributed beef breed in the world (Porter, 1991). Herefords have been exported since 1817, first to North America from where they spread to Mexico and South America. This breed and its crosses still dominate the beef herds of North and South America, Australia, and New Zealand. Furthermore, the Hereford has contributed to the formation and improvement of at least two dozen breeds across the world (Porter, 1991). For example, the Kazakh White-headed cattle breed was developed by crossing local cattle from Kazakhstan with Hereford cattle, imported from England and Uruguay between 1928 and 1932 (Porter, 1991). If Eu1 was distributed in Hereford beef cattle it has not remained within this breed; isolates from Korea and many of the isolates from the GB were from dairy cattle.

4.3. Secondary dispersal

The dispersal of Eu1 strains may be more complicated than a simple bovine diaspora from the UK. For example, in the Republic of Korea Eu1 strains were identified and Holstein cattle were imported to Korea from France in 1902; the first report of bovine TB in Korea was in 1913 (Wee et al., 2009). However, the most likely source of the Eu1 strains in Korea identified here are the many

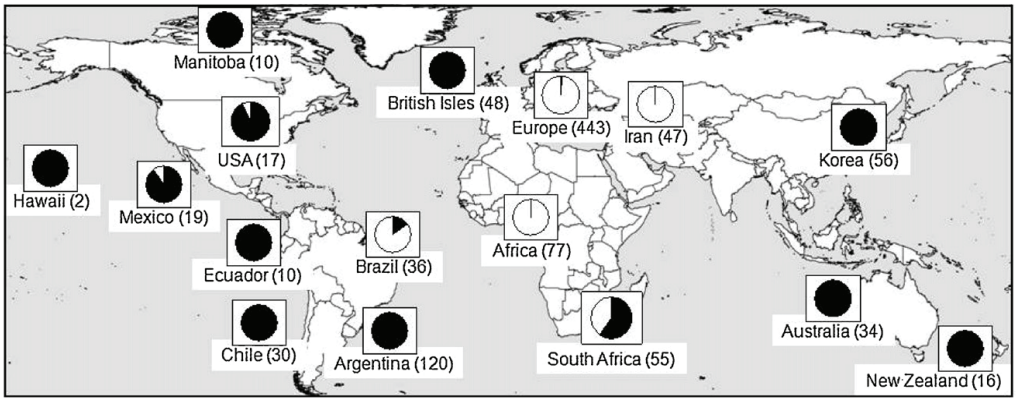

Fig. 2. Distribution of the Eu1 clonal complex of *M. bovis* in the countries surveyed. The pie charts show the proportion of isolates that are members of the Eu1 clonal complex; black = Eu1, white = other clonal complexes. The number of strains deletion typed for RDEu1 in each region are shown in parentheses. Eu1 strains have also been found in humans in Kazakhstan and Taiwan.

Holstein dairy cattle imported in the 1960s from USA, Canada, New Zealand and Australia, all countries where strains of the Eu1 clonal complex have been identified at high frequency (Bae, 1997; Wee et al., 2009). The Eu1 strains currently found in the Republic of Korea may represent the introduction of the disease from a source other than the UK and it is interesting to note the dominance of the SB0140 spoligotype pattern in both Australian and Korean isolates.

A complex history of cattle importation may even apply to the English speaking former British colonies. South Africa imported cattle not just from Europe but also from Argentina and Australia (Huchzermeyer et al., 1994). For both Australia and New Zealand, again, the introduction of the Eu1 clonal complex may not have been directly from the UK. The import of cattle to Australia has been recorded since the 1790s, however, these cattle were not primarily imported from the UK (Pierce, 1975). Between 1788 and 1825 cattle were imported from the, then, British colonies of India and South Africa. The initial import of cattle into New Zealand were from Australia in 1814 (Pierce, 1975). It was not until 1871 that Australia introduced a quarantine act to provide protection from various cattle diseases. However, the Custom Act of 1879 banned the import of cattle and sheep from all countries except GB and the RoI.

4.4. Why is Eu1 so common?

The simplest explanation for the global dominance of the Eu1 clonal complex is demography. Perhaps the Eu1 clonal complex was the lucky group of strains that happened to be distributed throughout the world as specialized breeds of cattle were exported from a single source and then re-exported between other countries.

However, an obvious explanation for the dominance of the Eu1 clonal complex over other strains of *M. bovis* is increased fitness such as reduced virulence (asymptomatic disease) or increase transmissibility. It is not clear to us that the RDEu1 deletion does convey such a fitness advantage; as discussed above the loss of *treY* function may be selectively neutral. Furthermore, just because a clonal complex is common does not necessarily imply that it has a fitness advantage. We note that strains of the Eu1 clonal complex have frequently become established in wildlife species: brush-tailed possums in New Zealand; white-tailed deer in Michigan, USA; wild boar in Hawaii, USA; badgers in GB and buffalo in South Africa. However, the ability to 'jump host' is not a unique characteristic for Eu1 strains; another clonal complex of *M. bovis* has established itself in wildlife in the Kruger National Park. It is more likely that the frequency of Eu1 strains in wildlife reflects the global prevalence of these strains worldwide, and thus an increased chance of spill over, rather than a specific attribute of this clonal complex.

4.5. Conclusion

The Eu1 clonal complex of *M. bovis* is common in many countries throughout the world (Fig. 2). Although the number of strains sampled was small for many countries we were, nonetheless, able to demonstrate the presence of Eu1 clonal complex strains. We do not have enough data to measure the ultimate importance of this clonal complex but it must constitute a significant proportion of the total bovine TB in the world. We are not convinced that Eu1 has a selective advantage over other clones of *M. bovis* and we suggest that simple demography might better explain the global distribution of Eu1; it was the lucky clone in the right place at the right time.

We note the association of the Eu1 clonal complex with countries that were formally part of the British Empire, yet, this is not a simple relationship. The Eu1 clonal complex is not at high frequency in the former British colonies of Nigeria, Uganda, and Tanzania (Berg et al., 2011; Muller et al., 2009) and we suspect that the global distribution of this clonal complex may be more complex than a simple dispersal from one country. Furthermore, it is entirely possible that the Eu1 clonal complex did not evolve in the UK but was imported into the UK from another country; in which case the UK may have merely been a distribution center for a clonal complex of bovine TB that evolved elsewhere. We note that Hereford beef cattle, bred in and distributed from the UK since the 19th century, would have provided a good vehicle for the global distribution of this clonal complex.

For the molecular epidemiologist the identification of clonal complexes provides a new tool in the analysis of otherwise large and intractable genotype datasets. In combination with geographical localisation of genotype, which is becoming an important observation for genotypes of *M. bovis*, the analysis of clonal complexes can be used to attribute imported strains to their International source. This has been done successfully with strains of the Af1 and Af2 clonal complexes isolated from humans in the UK and France and, in unpublished data, Eu1 strains in Italy were found in cattle recently imported from the British isles and thus given unequivocal attribution to source. However, and perhaps more important, the identification of clonal complexes is generating testable hypotheses that are a first step in understanding the phylogeography, demography and global distribution of this important veterinary pathogen

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.04.027.

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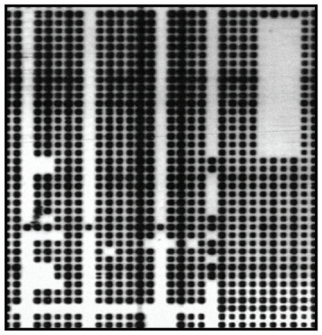
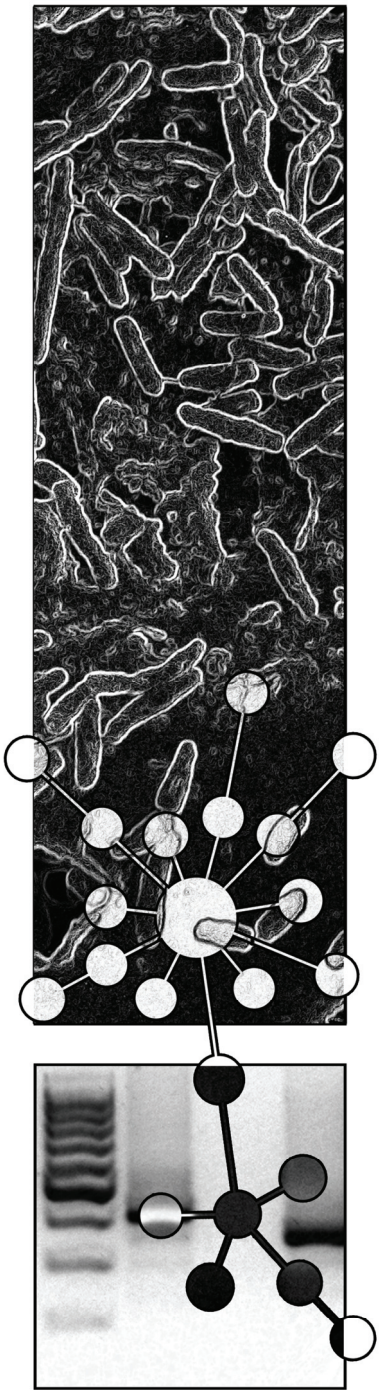
Table 12. Spanish *Mycobacterium bovis* strains deletion typed for RDEu1. Extract from the supplementary material available in the online version, at doi:10.1016/j.meegid.2011.04.027.

Lab. ID	Strain ID	Spoligotype	RDEu1	Spacer 11	Host	Country of isolation	Where isolated
1	03/1725	SB0121	intact	present	cattle	Spain	Toledo
2	04/1169	SB0121	intact	present	cattle	Spain	Cantabria
3	MI05/02585	SB0121	intact	present	cattle	Spain	Salamanca
4	MI05/04354	SB0121	intact	present	cattle	Spain	Navarra
5	MI05/04855	SB0121	intact	present	cattle	Spain	Jaen
6	MI06/00705	SB0121	intact	present	cattle	Spain	Barcelona
7	MI07/02929	SB0121	intact	present	cattle	Spain	Madrid
8	MI07/03957	SB0121	intact	present	cattle	Spain	Burgos
9	MI06/02524	SB0121	intact	present	cattle	Spain	Zamora
10	00/500	SB0121	intact	present	cattle	Spain	Madrid
11	MI07/00673	SB0121	intact	present	cattle	Spain	Salamanca
12	MI07/00706	SB0121	intact	present	cattle	Spain	Salamanca
13	MI07/03944	SB0121	intact	present	cattle	Spain	Avila
14	MI07/06622	SB0121	intact	present	cattle	Spain	Soria
15	MI07/05827	SB0121	intact	present	cattle	Spain	Salamanca
16	MI07/08687	SB0121	intact	present	cattle	Spain	Segovia
17	MI07/08696	SB0121	intact	present	cattle	Spain	Avila
18	MI07/04573	SB0121	intact	present	cattle	Spain	Cantabria
19	MI06/00631	SB0265	intact	present	cattle	Spain	Salamanca
20	MI06/05468	SB0265	intact	present	cattle	Spain	Burgos
21	MI06/05240	SB0265	intact	present	cattle	Spain	Jaen
22	MI07/08618	SB0265	intact	present	cattle	Spain	Salamanca
23	02/1081	SB0295	intact	present	cattle	Spain	Caceres
24	03/1742	SB0295	intact	present	cattle	Spain	Jaen
25	MI06/05461	SB0295	intact	present	cattle	Spain	Salamanca
26	MI06/00198	SB0120	intact	present	cattle	Spain	Zamora
27	MI06/04806	SB0120	intact	present	cattle	Spain	Salamanca
28	03/0243	SB0134	intact	present	cattle	Spain	Cantabria
29	MI06/07830	SB0134	intact	present	cattle	Spain	Salamanca
30	MI06/07667	SB0339	intact	present	cattle	Spain	Navarra
31	MI06/00370	SB0828	intact	present	cattle	Spain	Salamanca
32	00/311	SB0920	intact	present	cattle	Spain	Asturias
33	MI06/03860	SB0122	intact	present	cattle	Spain	Madrid
34	MI05/04392	SB1345	intact	present	cattle	Spain	Zamora
35	MI07/10865	SB1322	intact	present	cattle	Spain	Cordoba
36	01/395	SB0933	intact	present	cattle	Spain	Albacete
37	01/192	SB0933	intact	present	cattle	Spain	Albacete
38	MI05/04464	SB1308	intact	present	cattle	Spain	Madrid
39	MI07/01155	SB1398	intact	present	cattle	Spain	Cadiz
40	MI06/07666	SB0881	intact	present	cattle	Spain	Navarra
41	03/1103	SB1313	intact	present	cattle	Spain	Salamanca
42	MI06/04824	SB1369	intact	present	cattle	Spain	Salamanca
43	MI06/05473	SB1253	intact	present	cattle	Spain	Valladolid
44	03/0961	SB1309	intact	present	cattle	Spain	Cantabria
45	00/661	SB0807	intact	present	cattle	Spain	Asturias

Lab. ID	Strain ID	Spoligotype	RDEu1	Spacer 11	Host	Country of isolation	Where isolated
46	MI07/04925	SB1630	deleted	deleted	cattle	Spain	Orense
47	MI07/04910	SB1629	deleted	deleted	cattle	Spain	Orense
48	MI06/07062	SB1385	deleted	deleted	cattle	Spain	Lugo
50	MI06/00101	SB1357	deleted	deleted	cattle	Spain	Asturias
51	MI07/06616	SB1348	deleted	deleted	cattle	Spain	Palencia
52	MI07/02251	SB1312	deleted	deleted	cattle	Spain	Asturias
53	MI06/01259	SB1303	deleted	deleted	cattle	Spain	Burgos
54	99/644	SB1287	deleted	deleted	cattle	Spain	Asturias
55	00/258	SB1286	deleted	deleted	cattle	Spain	Asturias
56	MI05/03720	SB1164	deleted	deleted	cattle	Spain	Navarra
57	MI06/05796	SB1019	deleted	deleted	cattle	Spain	Lugo
58	MI05/00288	SB1016	deleted	deleted	cattle	Spain	Cantabria
59	MI07/03508	SB0140	deleted	deleted	cattle	Spain	Cordoba
60	MI07/08706	SB0140	deleted	deleted	cattle	Spain	Salamanca
61	99/323	SB0140	deleted	deleted	cattle	Spain	Asturias
62	MI05/00917	SB0130	deleted	deleted	cattle	Spain	Burgos
63	MI07/06449	SB0130	deleted	deleted	cattle	Spain	Ciudad Real
64	MI07/08543	SB1635	intact	deleted	cattle	Spain	Leon
65	MI06/07811	SB1387	intact	deleted	cattle	Spain	Salamanca
66	MI06/04843	SB1371	intact	deleted	cattle	Spain	Soria
67	MI05/03078	SB1353	intact	deleted	cattle	Spain	Girona
68	03/1499	SB1320	intact	deleted	cattle	Spain	Caceres
69	MI05/00297	SB1294	intact	deleted	cattle	Spain	Salamanca
70	99/590	SB1284	intact	deleted	cattle	Spain	Asturias
71	MI05/00414	SB1259	intact	deleted	cattle	Spain	Jaen
72	MI05/04830	SB1257	intact	deleted	cattle	Spain	Jaen
73	MI05/01273	SB1189	intact	deleted	cattle	Spain	Cantabria
74	MI06/01537	SB1075	intact	deleted	cattle	Spain	Huesca
75	MI05/01163	SB0853	intact	deleted	cattle	Spain	Cantabria
76	MI05/00284	SB0152	intact	deleted	cattle	Spain	Cantabria
77	MI06/05867	SB0152	intact	deleted	cattle	Spain	Lleida
78	MI05/04739	SB0130	deleted	deleted	red deer	Spain	Ciudad Real
79	MI07/02254	SB1312	deleted	deleted	badger	Spain	Asturias
80	MI07/03930	SB0140	deleted	deleted	wild boar	Spain	Soria





Discussion

In this thesis two approaches were used with the objective to improve our understanding of the molecular epidemiology of bovine and caprine tuberculosis in Spain. On the one hand, the standard molecular techniques spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing were applied for large-scale studies, on the other hand, specific methods including DNA microarray, whole genome sequencing and single nucleotide polymorphism (SNP) typing were exploited to delineate the phylogeny of *M. bovis* in Spain. In order to exploit the data obtained with the standard typing techniques a national database was created that reinforces the Spanish national programme of eradication of bovine tuberculosis in terms of molecular epidemiology.

The characterisation of 6215 Spanish *M. bovis* isolates from different animal host species and geographical regions by spoligotyping identified 252 different spoligotyping patterns and hence showed a high strain diversity which is similar to findings from continental Europe [France (156 *M. bovis* spoligotypes/1,349 isolates) (Haddad *et al.*, 2001) and Portugal (28/293) (Duarte *et al.*, 2008)], but different from Great Britain and Ireland [Great Britain (34/9,839) (Hewinson *et al.*, 2006; Smith *et al.*, 2006), Northern Ireland (14/461) (Skuce *et al.*, 2005) and the Republic of Ireland (20/452) (Costello *et al.*, 1999)]. Spoligotypes that accounted for at least 1% of the isolates were considered the most frequent types in Spain and spoligotype SB0121 (27.94% of the total of isolates) was clearly predominant. Most of the 15 more frequent spoligotypes were isolated throughout the surveyed years and from all over Spain, except from spoligotypes SB0135, SB1232 and SB1258 that were isolated during intensive sampling in certain areas. The most frequent patterns reveal common features between Spain and other western European countries with SB0121 being among the predominant patterns in France, Portugal and Italy (Haddad *et al.*, 2001; Duarte *et al.*, 2008; Boniotti *et al.*, 2009); moreover, SB0120 (BCG-like) (3.96% of the total of Spanish *M. bovis* isolates) is the most frequent type in France and Italy, but surprisingly infrequent among the Portuguese *M. bovis* population. The similarities between the Spanish, French, Portuguese and Italian *M. bovis* populations are probably caused by animal movements and trade relations. However, it is interesting that despite of Portugal's strong trade relations with the United Kingdom, the Spanish and Portuguese *M. bovis* populations are very similar. We also found similarities to strains from the United Kingdom and the Republic of Ireland where spoligotype SB0140 (1.01% of the total of Spanish *M. bovis* isolates) prevails and spoligotypes SB0130 and SB0134 (3.39% and 11.19% of our isolates, respectively) are also frequent (Costello *et al.*, 1999; Skuce *et al.*, 2005; Hewinson *et al.*, 2006; Smith *et al.*, 2006). SB0140 has also been described as the predominant spoligotype in earlier studies from South America (Zumarraga *et al.*, 1999) and Australia (Cousins *et al.*, 1998). Isolations of *M. bovis* with spoligotype SB0121 were reported with a negligible frequency (<1%) in Great Britain (Hewinson *et al.*, 2006). The

most frequent strains in Great Britain and Ireland have less spacers present in their spoligotyping profiles, which would imply that they descended from strains continental European strains.

Regarding the geographical distribution of the different spoligotypes at national level, we found that most of them had a wide distribution while only 33 types appeared localised. We also observed the isolation of new patterns when spoligotyping was extended to isolates from regions that had not been studied before. Interestingly, in the northern regions, that were the first to implement measures for the eradication of bovine tuberculosis, a higher diversity was found suggesting that the test-and-slaughter policy has not affected the strain diversity. In general, we could state that in provinces with lower herd prevalence (<0.8%) the indices of discrimination (D) (Hunter and Gaston, 1988; Hunter, 1990) showed slightly higher values (average D=0.90) compared to national average, whereas provinces with extreme herd prevalence (>4%) showed lower D values (average D=0.82). Nevertheless, exceptions might occur due to differences regarding cattle breeds and herd management. From the point of view of distribution by animal species, wildlife was shown to be clearly involved in the epidemiology of the *M. bovis* infection with 12 out of the 15 most frequent spoligotypes being present in cattle and at least in one wild artiodactyl which underlines the importance of wildlife, especially wild boar and red deer, as reservoir hosts in Spain (Aranaz *et al.*, 1996; Aranaz *et al.*, 2004; Hermoso *et al.*, 2006; Naranjo *et al.*, 2008; Gortázar *et al.*, 2011). The number of spoligotypes exclusively isolated from one animal species is notably higher in cattle (239 spoligotypes/207 exclusive to cattle) than in wild boar (26/6) or red deer (22/2) revealing that the infection in cattle brings about the strain diversity. Undoubtedly, the extensive farm management widely used in Spain should be considered a risk factor for the transmission from cattle to wildlife or vice versa (spillover and spillback) (Daszak *et al.*, 2000; Allepuz *et al.*, 2011).

Since the evolution of the direct repeat (DR) region is unidirectional, occurring by single spacer deletions or loss of contiguous spacer sequences (Fang *et al.*, 1998; van Embden *et al.*, 2000), we could consider SB0120 (BCG-like) the common ancestor, from which SB0121 has evolved by loss of spacer 21 giving rise to a diverse *M. bovis* population in which almost 70% of the isolates lack spacer 21. Cattle was domesticated at the beginning of the Neolithic (Beja-Pereira *et al.*, 2006; Edwards *et al.*, 2007) and studies based on mitochondrial DNA describe the spread of cattle from the Fertile Crescent to Europe following land and sea routes (Götherström *et al.*, 2005; Beja-Pereira *et al.*, 2006). According to these reports, cattle in the Iberian Peninsula also showed a strong influence of cattle of North African origin in contrast to cattle in eastern and central Europe. It is unknown whether the infection with *M. bovis* or rather an ancestral *M. bovis*-like strain with a maximum number of spacers has spread along with the

extension of cattle, as hypothesised for *M. tuberculosis* infection in humans (Wirth *et al.*, 2008; Hershberg et la., 2008), or has emerged later on infecting cattle populations that were naïve to tuberculosis. However, the diversity in the different cattle populations, for example autochthonous and bullfighting breeds, the differences in the holding systems and international trade have probably shaped the individual *M. bovis* populations in today's Europe.

A similar study was conducted on 791 *M. caprae* isolates obtained by sampling in many Spanish regions during sixteen years. The diversity among isolates of *M. caprae* was notably lower compared to *M. bovis* and spoligotyping yields an index of discrimination (D) of only 0.584. Additional MIRU-VNTR typing of 20 *M. caprae* isolates that originated from six goat flocks and four cattle farms with two different spoligotypes detected at a time confirmed that a change in the spoligotyping pattern is not necessarily linked to changes in the MIRU-VNTR type in agreement with previous findings in *M. bovis* (Romero *et al.*, 2008). At five farms the MIRU-VNTR types of the analysed pair of isolates were identical while the spoligotyping profile changed due to the loss of spacers 25 to 27, 29, and 34 to 38, which can occur in a single deletion event. Among the 791 *M. caprae* isolates, spoligotype SB0157 was the most frequently identified (25.8%) and moreover, it was present in all the animal species affected (goats, sheep, cattle, wild boar, red deer) except from fox (n=1; SB0416). Spoligotype SB0157 is the only strain of *M. caprae* reported from Portugal (Duarte *et al.*, 2008); the second most frequent spoligotype in this study, SB0416 (18.20%), has also been described in goats from Greece (Ikonomopoulos *et al.*, 2006). *M. caprae* caused 7.4% of the total of studied cases of animal tuberculosis. Surprisingly, cattle were involved in 53.8% of the outbreaks during the study period, and since 2004 a significant increase (p=0.009, by Mantel trend test) of bovine tuberculosis due to *M. caprae* has been noted, although the majority of *M. caprae*-infected cattle (86.7%) did not have direct contact to small ruminants. Detection of potential interspecies transmission is in accordance with other authors who described cattle-to-cattle transmission (Prodinger *et al.*, 2002; Pavlik *et al.*, 2002a; Erler *et al.*, 2004), possible cattle-to-pig and cattle-to-wild boar transmission (Pavlik *et al.*, 2002a; Erler *et al.*, 2004), and transmission between a zoo camel and a bison (Pate *et al.*, 2006). The menace that *M. caprae* may present to cattle is of relevance in virtually bovine tuberculosis free countries such as Germany, Austria, the Czech Republic and Hungary, where *M. caprae* is occasionally isolated from wildlife. *M. caprae* infection in wildlife in Spain has previously been reported (Parra *et al.*, 2003; Aranaz *et al.*, 2004b; Gortázar *et al.*, 2005) and also in this study we found the pathogen in several wildlife species: wild boar, wild deer and fox (first description in this species), all presenting gross lesions compatible with tuberculosis. If *M. caprae* is not thoroughly addressed within the eradication programmes, the emergence of this pathogen in wildlife might be observed, which would again threat the domestic animals by spillback

form the reservoir hosts. Since caprine tuberculosis is not included in the list of diseases notifiable to the OIE, it is not subjected to eradication campaigns. Nevertheless, it would be important to consider the extension of the eradication programmes to include goats in countries with an important goat population. In Spain, the current eradication programme considers small ruminants that co-exist with cattle in the same farm, and there are few local eradication programmes in goats (Murcia, Castilla y León, the Canary Islands and Andalucía), yet it would be advisable to address caprine tuberculosis at national level.

The sequencing of the 5' region of the DR of the three most prevalent *M. caprae* spoligotypes confirmed that it is highly homogeneous presenting the deletion of spacers 1, and 3 to 16; these deletions comprised the entire DR and the adjacent spacer as described by van Embden (2000). Interestingly, all Spanish *M. caprae* isolates lacked spacers 30 to 33, except from three cattle isolates with spoligotypes SB0418 and SB1619. These animals had been imported to Spain from Eastern Europe where strains with spacers 30 to 33 are common (Pavlik *et al.*, 2002a; Lantos *et al.*, 2003; Erler *et al.*, 2004; Prodinger *et al.*, 2005; Jánosi *et al.*, 2009). Spoligotype SB0418 has frequently been reported from cattle and wildlife in Central and Eastern European countries (Haddad *et al.*, 2001; Pavlik *et al.*, 2002a; Pavlik *et al.*, 2002b; Erler *et al.*, 2003; Erler *et al.*, 2004), Italy (Boniotti *et al.*, 2009), and also caused an outbreak in a Slovenian zoo (Pate *et al.*, 2006). Furthermore, *M. caprae* spoligotype SB0418 has caused human tuberculosis in Germany (Blaas *et al.*, 2003), as well as SB1690 in Slovenia (Erler *et al.*, 2004). The observation of a spoligotype signature (loss of spacers 30 to 33) for the Iberian cluster of *M. caprae* suggests that this cluster has evolved from eastern and central European strains. The broad host range of *M. bovis* and *M. caprae* reflects the ability of these pathogens to survive in different hosts. Their importance as potential human pathogens is reflected by a recent study that describes the most prevalent spoligotypes in animals, *M. bovis* SB0121 and *M. caprae* SB0157, as the most frequently isolated strains from human tuberculosis due to other than *M. tuberculosis* (Rodríguez *et al.*, 2009).

The review of the literature reveals that in every country there is one clearly prevalent spoligotype, like SB0121 in Spain. Therefore, the study of *M. bovis* isolates by spoligotyping raised the question whether spoligotype SB0121 has an increased fitness or spoligotyping was unable to subtype these strains. In order to evaluate the potential of MIRU-VNTR typing to further discriminate the most frequent spoligotype in Spain, SB0121, a panel of 115 isolates was studied using nine MIRU-VNTR markers. The isolates were subtyped into 65 different MIRU-VNTR types yielding an index of discrimination (D) of 0.9856. This result is similar to findings in Portugal using MIRU-VNTR typing with eight loci (D=0.96) (Duarte *et al.*, 2010) and combined spoligotyping and eight-loci MIRU-VNTR typing (D=0.99) (Cunha *et al.*, 2011), and to a report from Italy where a

discrimination of 0.987 was achieved using 13 loci for the typing of *M. bovis* spoligotype SB0120 (Boniotti *et al.*, 2009). Minimum spanning trees were constructed for combinations with nine loci, seven loci and four loci, and in all cases the majority of the genotypes (87.7% in the case of the nine-loci approach) were closely related resulting in the expansion of a clonal group when the maximum difference within a clonal complex was set to two loci (double locus variant = DLV). No geographic clustering of genotypes could be observed, in contrast to a report from the UK (Smith *et al.*, 2003; Smith *et al.*, 2006b), where bovine tuberculosis was almost eradicated and then reemerged in the mid-1980s. We could hypothesise that the increased diversity in Spain might be due to "old" strains that have been circulating for a longer time.

An identical situation was revealed in the attempt to trace back the first case of tuberculosis in alpacas in Spain that was caused by *M. bovis* spoligotype SB0295, the fifth most frequent spoligotype in Spain (4.1%) and the most prevalent in Andalucía (18.7%). Forty-seven isolates were selected as potential source and typed using nine MIRU-VNTR markers resulting in 22 different genotypes (combined spoligotype and MIRU-VNTR type); none of these matched the genotype of the causative agent of the alpaca tuberculosis. Yet, in a population snapshot performed by eBURST V3 (Spratt *et al.*, 2004) all but five of the genotypes were closely related and grouped into a clonal group and a linked cluster made up by single allele variants (SLV). The putative founder of the clonal group was the most abundant genotype that was found throughout the years and all over the studied region; this strain could have given rise to the variety of genotypes. Despite the fact that no wildlife isolates of spoligotype SB0295 or related spoligotyping patterns were known at the time of the study, wildlife as a source of infection should not be ruled out since wild boar and other ungulates are overabundant in southern Spain and only few biosafety measures (e.g. fencing) are taken (Ballesteros *et al.*, 2009; Muñoz *et al.*, 2010). That underlines the importance and necessity of centralising molecular typing data at national level. We concluded that in a high diversity setting such as the Iberian Peninsula genotyping data have to be interpreted against the background of a possible clonal expansion, taking into account related isolates [SLVs, DLVs or even triple locus variants (TLVs)] when investigating potential sources.

The most discriminatory loci for the *M. bovis* isolates with spoligotype SB0121 were QUB3232 (D=0.83), ETR-A (D=0.65) and ETR-B (0.53) followed by QUB11a (D=0.45), QUB26 (D=0.38) and MIRU26 (D=0.35). The loci ETR-D, ETR-E and QUB11b tailed the field with low allelic diversities of 0.20, 0.17 and 0.08, respectively; however, the loci ETR-D and ETR-E, together with MIRU26, are convenient for MIRU-VNTR typing of *M. caprae* isolates (Prodinger *et al.*, 2005) and therefore these loci were not excluded from the selected marker set. These observations are comparable to previous studies on *M. bovis*

(Allix *et al.*, 2006; Boniotti *et al.*, 2009; Duarte *et al.*, 2010), except from QUB11b that was rated to have an intermediate discrimination. Interestingly, variations of the allelic diversities of some MIRU-VNTR markers varied depending on the selection of strains, such as in the case of QUB11b that was performing poorly in the typing of spoligotype SB0121 isolates and in the selected set for tracing the outbreak of alpaca tuberculosis, while it was among the four most diverse loci for the panel of isolates from bullfighting cattle, or also ETR-B that was highly diverse for isolates with spoligotype SB0121, achieved intermediate discrimination in the set of isolates from bullfighting cattle, but only low discrimination in the selected set for tracing the outbreak of alpaca tuberculosis. Furthermore, it needs to be noted that not all spoligotypes may be subtyped to such a large extent than SB0121, as can be observed for spoligotype SB0295 that showed less variation. Loci QUB3232 and QUB11a have been described as hypervariable due to varying results in epidemiologically linked isolates and in serial isolations from the same patient, and are therefore not included in the standard marker set for typing *M. tuberculosis* (Supply *et al.*, 2006). Notwithstanding, several authors suggested the usefulness of these markers with regard to a determined setting (Hilty *et al.*, 2005), lineage (Velji *et al.*, 2009) or mycobacterial species, such as *M. bovis* (McLernon *et al.*, 2010; Lari *et al.*, 2011). In our studies we could not confirm the hypervariability of these two loci. Although QUB3232 repeatedly showed the highest diversity, we did not find that it caused differentiation of epidemiologically linked isolates to a significantly higher degree than other loci, and when excluding QUB3232 and QUB11a from the analysis we still observed a clonal expansion of strains without clear geographical associations. In fact, the use of these two loci together with ETR-A and ETR-B led to a satisfactory discrimination (D=0.97) of the set of spoligotype SB0121 isolates and thus could reduce the time and cost expenditure of MIRU-VNTR typing. A standardised combination of loci would be desirable and in fact, a combination of six markers (ETR-A, ETR-B, ETR-D, QUB11a, QUB11b and QUB3232) has been proposed by VENoMYC for the MIRU-VNTR typing of *M. bovis* strains (EU coordination action SSPE-CT-2004-501903; Supply, 2006), but the differences between the countries regarding the allelic diversities of the different MIRU-VNTR loci lead to individualised marker combinations.

The results obtained with spoligotyping and MIRU-VNTR typing of *M. bovis* isolates from bullfighting cattle and wildlife on the same premises confirmed the circulation of identical or closely related genotypes within and between bullfighting herds and wildlife. It is noteworthy that seven of the spoligotypes from bullfighting cattle belonged to the fifteen most frequent types in Spain and that the other seven types could have derived from the more frequent spoligotypes in a single deletion event, or two deletion events in the case of one spoligotype. This is in accordance with Barret and colleages (2004) who stated that ongoing outbreaks with continued transmission are more likely to present genetic diversity than are point source outbreaks. A recent 12-

year based study on herd-transmission dynamics in Spain reports that the control of infection with tuberculosis takes longer in extensive herd types (beef and bullfighting herds), prevalent in Southern Spain, than in dairy cattle (J. Álvarez, submitted manuscript) which underlines the possibility of evolution of the spoligotypes.

The importance of molecular typing and the centralisation of the corresponding data at national level has been recognised since the implementation of typing techniques in the 1990s and is reflected in the studies comprised in this thesis. The Spanish Ministry of the Environment, and Rural and Marine Affairs (MARM), supported by the Regional Laboratories, aimed at centralising the Spanish molecular typing data obtained by spoligotyping, which is applied routinely to all *M. tuberculosis* complex isolates, and MIRU-VNTR typing, which is conducted in selected sets of isolates, in order to intensify the national eradication programme of bovine tuberculosis that has reduced the herd prevalence from 2.11% to 1.51% in the last decade (MARM, 2010). The VISAVET Centre was commissioned this task and a database, mycoDB.es, was created and set up within this PhD project. However, it should be recognised that the collection of samples and data resulted from the involvement of numerous contributors, which reflects the commitment at national level on the study of the epidemiology of bovine tuberculosis and the successful collaboration between researchers and administration, which is indispensable for the progress in the eradication campaign (Task Force Bovine Tuberculosis Subgroup, 2006; Proceedings of the 4th International Conference on *Mycobacterium bovis*, 2006). A large amount of typing data consisting of 17273 spoligotyped isolates out of which 410 were additionally MIRU-VNTR typed was centralised in an intuitive and hence user-friendly database named mycoDB.es that enables inquiries at large-scale and local level. The centralisation of molecular data has increased our knowledge of the demography of *M. bovis* and *M. caprae* in a broad context and also corroborated transmission of these pathogens between wildlife and domestic animals. The database could be improved by providing it with a geographical information system (GIS) that would allow the study of small-scale transmission between neighbouring farms or between animals on communal pastures. Furthermore, it is important to keep in mind that the search option is on perfect match basis, which excludes possible closely related genotypes, for example due to deletion of a direct variant repeat (DVR) or block of DVRs or to changes in the MIRU-VNTR type at a single locus. Therefore molecular data should always be combined with the classical epidemiological information. At long sight mycoDB.es could hint isolates of atypical behaviour, such as emerging strains with increased clonal expansion or hypervariable isolates. Members of the *M. tuberculosis* complex also pose a serious risk to human health and the mycoDB.es database can simplify the data exchange between public health and veterinary services.

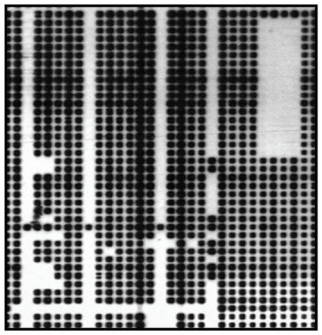
The application of standard typing methods has been extensively discussed and the study of the molecular epidemiology of the Spanish *M. bovis* population has revealed common features as well as marked differences among the isolates at national and international level. These characteristics led to the collaboration in phylogenetic studies involving numerous institutions from over 30 countries. A selection of 20 Spanish *M. bovis* isolates with spacers 3 to 7 missing in their spoligotyping pattern was screened for the absence of the Region of Difference (RD) African 2 (Af2). The absence of both, the block of spacers and RDAf2, defines the clonal complex African 2 predominant in East Africa (Berg *et al.*, 2011). The absence of spacers 3 to 7 in the Spanish isolates was due to homoplasy since RDAf2 was present in all isolates and thus these isolates were not members of the Af2 clonal complex. A different clonal complex of *M. bovis*, European 1 (Eu1), is at virtual fixation in the British Isles and widespread all over the globe (Smith *et al.*, 2011). This clonal complex is marked by the absence of spacer 11 and the deletion of RDEu1. Eighty Spanish *M. bovis* isolates were selected for deletion typing of RDEu1 and the maximum of estimated Eu1 strains in the Spanish population was low (6.1%), similar to Portugal (7.6%). Clear signs of homoplasy were detected in 14 isolates that lacked spacer 11 from their spoligotype patterns but were intact at RDEu1.

The availability of molecular techniques such as microarray and whole genome sequencing has increased the number of studies regarding the demography of members of the *M. tuberculosis* complex and we were able to describe a new clonal complex of *M. bovis*, European 2 (Eu2), prevalent in Western Europe and marked by the loss of spacer 21 plus a mutation in the gene *guaA*. A survey of representative collections of *M. bovis* isolates from Spain, Portugal, Italy and France by SNP typing revealed that this clonal complex was dominant in the Iberian Peninsula [Portugal (74%), Spain (66%)] but less frequent in the other two countries [France (20.4%), Italy (1.6%)]., the presence of spacer 21-deleted spoligotypes is rare. In the British Isles and 99% of the strains belong to the Eu1 clonal complex (Smith *et al.*, 2011). Thus, strains of the Eu2 clonal complex are probably rare or absent from the British Isles. The multidrug-resistant *M. bovis* isolate with spoligotype SB0426 that caused the most important outbreak due to *M. bovis* in humans (Rullán *et al.*, 1996; Guerrero *et al.*, 1997; Rivero *et al.* 2001) is also marked by the absence of spacer 21 in the spoligotype pattern and the SNP in *guaA* and thus belongs to the Eu2 clonal complex (S. Samper, personal communication).

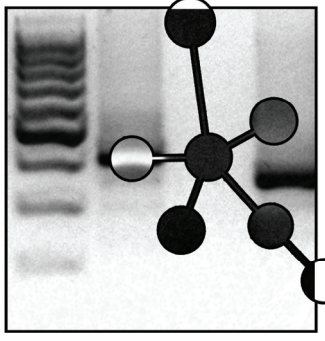
It is important to underline that spoligotype signatures (Streicher *et al.*, 2007) are useful to indicate a possible membership of a clonal complex but do not define a clonal complex per se (Smith *et al.*, 2011), since identical spoligotypes in unrelated strain lineages may occur as a result of convergent evolution (Warren *et al.*, 2002). Spoligotyping signatures have proven useful in earlier studies of the African 1 (Af1) clonal complex of *M. bovis* (Müller *et al.*, 2009) and also for subclones of *M. tuberculosis*

(Kato-Maeda *et al.*, 2011). In this study DNA microarray revealed a high homogeneity among the Spanish strains, and no phylogenetically informative deletion could be identified, in contrast to the previous studies and whole genome sequencing was needed to identify a suitable marker. The selection of a suitable phylogenetic marker, such as the SNP in *guaA*, needs to be made against the background of stability and essentiality of the affected genomic region, since deletions in regions with a high mutation rate may be prone to deletion, for example nonessential genes or regions with repetitive DNA. More studies on the SNPs identified in the Spanish strains by whole genome sequencing will be needed to define other suitable phylogenetic markers that could further delineate the phylogeny of the Spanish *M. bovis* isolates.

The mutation at *guaA* was not observed in reference strains of previously described clonal complexes Af1, Af2 and Eu1, suggesting that the Eu2 clonal complex forms a separate branch in the *M. bovis* phylogeny. However, a comparison of the three whole genome sequences of Spanish strains to the sequences of Af1 and Af2 strains would be interesting to assess the differences between the clonal complexes at SNP level. Further studies by whole genome sequencing are needed to elucidate whether the Eu2 strains are descendants or ancestors of the Italian or French *M. bovis* population. Possible members of the Eu2 clonal complex are also present in countries in South America and South Africa (Zumárraga *et al.*, 1999; Michel *et al.*, 2008) where they possibly have been introduced through international trade. Unpublished data confirmed three members of the Eu2 clonal complex in the Kruger National Park in South Africa (isolates provided by A. Michel via N.H. Smith). It would be interesting to further estimate the presence of the Eu2 clonal complex on other continents. The knowledge about clonal complexes and their distribution might be able to help us date the introduction of the disease in each country and may contribute to the identification of possible sources of tuberculosis and underlying reasons for the spread of *M. bovis* (Smith, 2011).



Conclusions



1. Spoligotyping revealed a high degree (D=0.87) of strain diversity among Spanish *Mycobacterium bovis* isolates and hence is a useful tool for the study of molecular epidemiology of the infection in our country. Nevertheless, the five most frequent spoligotypes of *M. bovis* are SB0121, SB0134, SB0339, SB0265 and SB0295, which account for more than 50% of the isolates. The most frequent spoligotypes are present all over the national territory and in domestic animal species as well as in wildlife.

2. The diversity of *Mycobacterium bovis* strains is higher in cattle than in other domestic and wild animal species, suggesting that the disease is maintained in cattle. The strain diversity also varies between geographical regions, and it seems not to be affected by the test-and-cull policy.

3. *Mycobacterium caprae* is the main causative agent of caprine tuberculosis which is widespread in Spain. However, it also poses a health risk to other domestic animals, wildlife and humans. The most frequent spoligotype of *M. caprae*, SB0157, clusters over 40% of the isolates.

4. Cases of bovine tuberculosis due to *Mycobacterium caprae* have increased significantly during the last years. Although this finding suggests that the pathogen is capable of circulating within and between cattle herds, it would be important to consider the implementation of a national eradication programme in caprine flocks, since goats are the main reservoir of this pathogen.

5. *Mycobacterium caprae* isolates from Spain show a specific spoligotype signature with spacers 30 to 33 absent from their spoligotyping pattern, in contrast to *M. caprae* strains from Central and Eastern Europe. The existence of a special feature of the Iberian cluster of *M. caprae* enables the identification of outbreaks over Europe.

6. The most frequent *Mycobacterium bovis* spoligotype in Spain, SB0121, presents a high diversity of mycobacterial interspersed repetitive unit-variable number repeat (MIRU-VNTR) types and expands as a cluster of closely related clonal groups.

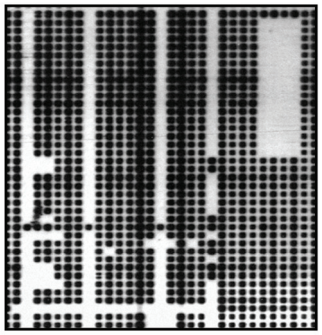
7. Due to the high diversity of the Spanish *Mycobacterium bovis* population a less stringent interpretation of the mycobacterial interspersed repetitive unit-variable number repeat (MIRU-VNTR) typing could be applied when screening for possible outbreak sources.

8. The combination of loci ETR-A, ETR-B, QUB11a and QUB3232 is a recommendable set of mycobacterial interspersed repetitive unit-variable number repeat (MIRU-VNTR) markers for typing Spanish *Mycobacterium bovis* isolates. For more specific

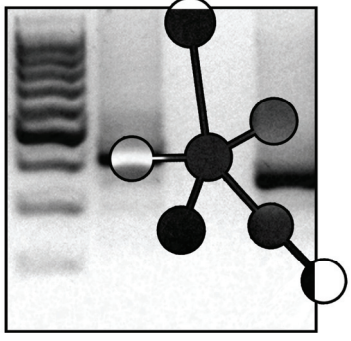
purposes, the following five loci might be analysed additionally: ETR-D, ETR-E, MIRU26, QUB11b and QUB26.

9. The national database mycoDB.es is a valuable tool for animal tuberculosis epidemiology and forms one of the pillars of the current national eradication programme for bovine tuberculosis in Spain.

10. The loss of spacer 21 from the spoligotyping pattern and a nucleotide change in gene *guaA* characterise the European 2 clonal complex of *Mycobacterium bovis* dominant in the Iberian Peninsula. In Spain, approximately 70% of the *M. bovis* isolates belong to the Eu2 clonal complex, while members of this lineage are less frequent in France and Italy, and absent from the British Isles. Members of the clonal complex European 1 are infrequent in Spain, and no member of the African 2 clonal complex was identified.



Summary



The eradication of bovine tuberculosis is indispensable due to its negative impact on animal health and welfare, and national as well as international trade. Moreover, the infection threatens endangered animal species and humans. *Mycobacterium* (*M.*) *bovis* and *M. caprae* are the main causative agents of animal tuberculosis in Spain. Although the cattle herd prevalence has been drastically reduced in the last decade (1.15%, 2010), bovine tuberculosis remains a problem. The use of typing techniques contributes to a better control and understanding of the molecular epidemiology of the disease and thus backs up the national eradication programme. The present thesis is structured in four independent chapter. In the first three chapters, the two molecular standard techniques direct variable spacer oligonucleotide typing (DVR-spoligotyping) and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) were applied to study the strain diversity, geographical distribution as well as the implication of wildlife in Spain.

Chapter I contains large population surveys of *M. bovis* and *M. caprae* in Spain by DVR-spoligotyping. The 6215 *M. bovis* isolates analysed yielded 252 different spoligotypes showing a high spoligotype diversity similar to other countries in continental Europe, but in contrast to the low diversity observed in the United Kingdom and the Republic of Ireland. The 791 *M. caprae* isolates clustered in only 15 spoligotypes. The most prevalent spoligotypes were *M. bovis* SB0121 and *M. caprae* SB0157. Both pathogens were widespread in Spain, with the most frequent strains being present in domestic animals and wildlife. Interestingly, an increase of cases of bovine tuberculosis due to *M. caprae* has been observed during the last years. These large population surveys hinted at two spoligotype signatures: the absence of spacer 21 from *M. bovis* spoligotyping profiles in 67% of the population, and the absence of spacers 30 to 33 from *M. caprae* spoligotyping patterns contrasting with the strains described in Central and Eastern Europe.

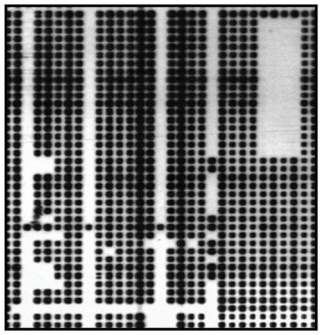
The MIRU-VNTR typing of three sets of selected isolates of *M. bovis*, which revealed an expansion of closely related clonal groups of the prevalent spoligotypes SB0121 and SB0295, are comprised in chapter II. The different sets included isolates with the spoligotype SB0121, a selection from a geographically limited area to track an outbreak on two alpaca farms, and a selection of isolates from bullfighting cattle and wildlife sharing their habitat. The fact that MIRU-VNTR types seem to evolve quickly in Spain might lead to difficulties with the identification of possible outbreak sources as experienced in the case of the first alpaca outbreak in Spain; hence, a less stringent comparison of MIRU-VNTR types might be employed for specific spoligotypes or geographical settings.

Chapter III describes the national database of animal tuberculosis, mycoDB.es,

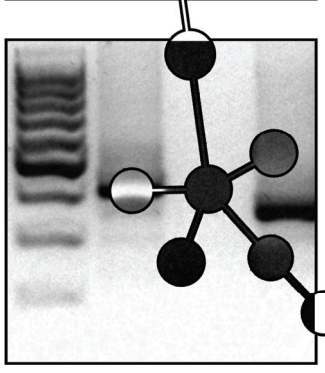
which has been designed as a tool within the Spanish programme of eradication of bovine tuberculosis. The access to the database is restricted to Official Veterinary Services and currently offers 17273 spoligotyping data out of which 410 are additionally MIRU-VNTR typed.

Chapter IV of this dissertation addresses the phylogeny of Spanish *M. bovis* isolates. For this purpose DNA microarrays, whole genome sequencing and single nucleotide polymorphism (SNP) typing were applied. A new clonal complex of *M. bovis* was defined based on the absence of spacer 21 from the spoligotyping pattern and a synonymous SNP in *guaA*. This clonal complex, named European 2 (Eu2), is prevalent in Spain and Portugal, less frequent in France and Italy and absent from the British Isles. The screening of Spanish *M. bovis* isolates with spoligotype signatures hinting at the African 2 (Af2) or the European 1 (Eu1) clonal complex revealed that no Af2 strains were present in the Spanish populations and that the proportion of Eu1 strains is estimated to be low.

In conclusion, the findings of this PhD thesis improved not only our understanding of the molecular epidemiology of *M. bovis* and *M. caprae* at national level and in the European context, but also the exploitation of molecular data and thus, contributed to a progress of the national eradication campaign.



Resumen en español



Objetivos y organización de la tesis

La erradicación de la tuberculosis bovina es una importante meta a nivel europeo y los Estados Miembro de la UE son responsables del diseño y de la ejecución de programas nacionales de erradicación. *M. bovis* y *M. caprae* son los agentes causantes de la tuberculosis en animales en España y no solo afectan al ganado vacuno y caprino, sino a un amplio abanico de especies animales y a los humanos. Para mejorar la campaña nacional de erradicación es necesaria una mejor comprensión de la epidemiología de estos patógenos. En España se reconoce la importancia tanto de la tuberculosis bovina como caprina, y el papel de la fauna salvaje como reservorio de la enfermedad se considera crucial para el éxito del programa de erradicación. Desde la implementación de técnicas de tipificación molecular la epidemiología molecular se ha convertido a nivel mundial en uno de los pilares de los programas de erradicación contribuyendo al mejor entendimiento y vigilancia de la enfermedad.

El objetivo global de la presente tesis doctoral es la aplicación de técnicas de tipificación molecular a nivel nacional con el fin de evaluar la situación epidemiológica en España en el contexto europeo. Por este motivo se han abordado varios objetivos presentados de manera independiente en los siguientes capítulos:

Capítulo I:	Demografía molecular de *Mycobacterium bovis* y *Mycobacterium caprae* en España
Capítulo II:	La tipificación molecular como herramienta para trazar brotes causados por *Mycobacterium bovis*
Capítulo III:	La base de datos nacional española de tuberculosis en animales - mycoDB.es
Capítulo IV:	La filogenia de *Mycobacterium bovis* en la Península Ibérica

Capítulo I: Demografía molecular de *M. bovis* y *M. caprae* en España

El objetivo de este capítulo fue el estudio a gran escala de aislados españoles de *M. bovis* y *M. caprae* por espoligotipado. El espoligotipado se ha aplicado en España durante los últimos 15 años, no obstante no se había realizado ningún estudio a nivel nacional desde 1996 para determinar el grado de diversidad en la población española de *M. bovis* y *M. caprae* y para comparar la situación en España con la de otros países europeos.

De estos estudios derivaron dos artículos científicos:

- Rodríguez, S., B. Romero, J. Bezos, L. de Juan, J. Álvarez, E. Castellanos, N. Moya, F. Lozano, S. González, J. L. Sáez-Llorente, A. Mateos, L. Domínguez, y A. Aranaz., y la Red Española de Vigilancia de la Tuberculosis en Animales. 2010. **High spoligotype**

diversity within a *Mycobacterium bovis* population: Clues to understanding the demography of the pathogen in Europe (Alta diversidad de espoligotipos en una población de *Mycobacterium bovis*: Claves para entender la demografía del patógeno en Europa). Veterinary Microbiology 141:89-95.

- Rodríguez, S., J. Bezos, B. Romero, de Juan L., J. Álvarez, E. Castellanos, N. Moya, F. Lozano, M. T. Javed, J. L. Sáez-Llorente, E. Liébana, A. Mateos, L. Domínguez, and A. Aranaz, y la Red Española de Vigilancia de la Tuberculosis en Animales. 2011. ***Mycobacterium caprae* infection in livestock and wildlife, Spain** (Infección por *Mycobacterium caprae* en animales domésticos y salvajes, España). Emerging Infectious Diseases 17:532-535.

Asimismo, se presentaron las siguientes contribuciones en congresos y reuniones de proyectos europeos:

- Rodríguez, S., A. Aranaz, B. Romero, L. de Juan, J. Bezos, J. Álvarez, E. Castellanos, N. Moya, F. Lozano, A. Mateos y L. Domínguez. **Spoligotyping diversity of *Mycobacterium bovis* in Spain** (Diversidad de espoligotipado de *Mycobacterium bovis* en España). Presentación oral. Taller "VNTR/MIRU and DVR-spoligotyping for *Mycobacterium bovis* typing" del Proyecto Europeo SSPE-CT-2004-501903. Toledo (España), 19-21 Octubre 2006.

- Rodríguez, S., E. Castellanos, J. Bezos, A. Aranaz, L. de Juan, F. Lozano, A. Mateos y L. Domínguez. **The usefulness of DVR-spoligotyping in characterizing Spanish isolates of the zoonotic agents *Mycobacterium bovis* and *Mycobacterium caprae*** (La utilidad del DVR-espoligotipado para la caracterización de aislados españoles de los agents zoonósicos *Mycobacterium bovis* y *Mycobacterium caprae*). Póster. 3rd Med-Vet-Net Annual Scientific Meeting. Lucca (Italia), 27-30 Junio 2007.

- Rodríguez, S., J. Bezos, L. de Juan, B. Romero, J. Álvarez, E. Castellanos, S. González, J. L. Sáez, A. Mateos, L. Domínguez y A. Aranaz. **Molecular epidemiology underlines the importance of *Mycobacterium caprae* in livestock and wildlife** (Epidemiología molecular resalta la importancia de *Mycobacterium caprae* en el ganado y la fauna salvaje). Presentación oral. 31st Annual Congress of the European Society of Mycobacteriology. Bled (Eslovenia), 4-7 Junio 2010.

Además se realizaron estudios adicionales incluidos por su temática en este capítulo:

- La distribución global de espoligotipos. Revisión bibliográfica basada en perfiles de *M. bovis* y *M. caprae* incluidos en la base de datos www.mbovis.org.

- Arboles filogenéticos de los espoligotipos presentes en España.

Capítulo II: La tipificación molecular como herramienta para trazar brotes causados por *M. bovis*

El objetivo de este capítulo fue la evaluación de la técnica de tipificación por VNTR en varios paneles seleccionados de aislados de *M. bovis*. En los últimos años la técnica de VNTR ha ganado en importancia para la trazabilidad de brotes de tuberculosis. Los estudios en los que se utilizó la tipificación por VNTR relatan la necesidad de considerar la región geográfica para definir una combinación de marcadores capaz de generar los mejores resultados. En este capítulo se estudió la diversidad alélica de varios marcadores y el poder discriminatorio global de la tipificación por VNTR en diferentes paneles de aislados de *M. bovis* y *M. caprae* para determinar una combinación conveniente de loci para la tipificación de aislados españoles.

Los estudios comprendidos en este capítulo resultaron en una publicación científica y un manuscrito:

- Rodriguez-Campos, S., A. Aranaz, de Juan L., J. L. Sáez-Llorente, B. Romero, J. Bezos, A. Jiménez, A. Mateos, y L. Domínguez. 2011. **Limitations of spoligotyping and variable number tandem repeat typing for molecular tracing of *Mycobacterium bovis* in a high diversity setting** (Limitaciones del espoligotipado y del análisis del número de repeticiones en tándem en la trazabilidad molecular de *Mycobacterium bovis* en un entorno de alta diversidad). Journal of Clinical Microbiology. 49:3361-3364.

- Rodriguez-Campos, S., B. Romero, L. de Juan, J. Bezos, A. Mateos, L. Domínguez y A. Aranaz. **Discrimination of variable number repeat typing rises with the expansion of a clonal group of *Mycobacterium bovis*** (La discriminación por análisis del número de repeticiones en tándem aumenta con la expansión de un grupo clonal de *Mycobacterium bovis*). Manuscrito en preparación.

La siguiente colaboración en un artículo científico derivó de estos estudios:

- García-Bocanegra, I., I. Barranco, I. M. Rodríguez-Gómez, B. Pérez, J. Gómez-Laguna, S. Rodríguez, E. Ruiz-Villamayor y A. Perea. 2010. **Tuberculosis in alpacas (*Lama pacos*) caused by *Mycobacterium bovis*** (Tuberculosis en alpacas [*Lama pacos*] causada por *Mycobacterium bovis*). Journal of Clinical Microbiology. 48:1960-1964.

Los resultados fueron presentados en los siguientes congresos y reunions de proyectos europeos:

- Rodríguez, S., E. Castellanos, L. de Juan, J. Bezos, F. Gallardo, N. Moya, J. Álvarez, N. Álvarez, T. Alende, A. Gutiérrez, F. Lozano, A. Mateos y B. Romero. **Mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) typing of**

SB0121, the most frequent spoligotype in Spain [Tipificación por repeticiones intercaladas en el genoma de las micobacterias y número variable de repeticiones en tandem (MIRU-VNTR) de SB0121, el espoligotipo más frecuente en España]. Presentación oral. Taller "VNTR/MIRU and DVR-spoligotyping for *Mycobacterium bovis* typing. Results of the VNTR-DVR Spoligotyping Ring Trial." del Proyecto Europeo SSPE-CT-2004-501903. Madrid (España), 24-25 Marzo 2009.

- Rodríguez, S., A. Aranaz, J. Bezos, E. Castellanos, L. de Juan, F. Gallardo, A. Gutiérrez, A. Mateos, L. Domínguez. y B. Romero. **High discrimination of the MIRU-VNTR technique for the most frequent spoligotype in Spain** (Alta discriminación por la técnica MIRU-VNTR del espoligotipo más frecuente en España). Presentación oral. *M. bovis* V Conference. Wellington (Nueva Zelanda), 25-28 Agosto 2009.

- Rodríguez, S. **Advances in Workpackage 6: Molecular characterisation of *M. bovis* and *M. caprae* isolates focused on epidemiological investigation** (Avances en el Workpackage 6: Caracterización molecular de aislados de *M. bovis* y *M. caprae* enfocada a la investigación epidemiológica). Reunión del Proyecto Europeo FP7-KBBE-2007-212414. Madrid (España). 11-12 Noviembre 2010.

Asimismo, otro estudio se engloba dentro de este capítulo:

- Análisis de un panel de aislados de *M. bovis* procedentes de ganado de lidia mediante espoligotipado y tipificación por VNTR. Manuscrito en preparación.

Capítulo III: La base de datos nacional española de tuberculosis en animales - mycoDB.es

El capítulo III hace una contribución directa al Programa Nacional de Erradicación de la tuberculosis bovina - la base de datos nacional de tuberculosis "mycoDB". Cuando el espoligotipado se convirtió en una técnica estándar dentro de la campaña nacional, la centralización de datos se volvió indispensable; por este motivo, una base de datos fue diseñada y puesta a punto en colaboración con el Ministerio de Medio Ambiente y Rural y Marino (MARM). Más adelante la base de datos fue ampliada para la inclusion de datos de la tipificación por VNTR. El acceso a los datos es concedido por el MARM a los Servicios Veterinarios oficiales y los Laboratorios Regionales. El conocimiento de la diseminación de los diferentes espoligotipos intra- e interespecies, y también geográfica, es imprescindible para observer relaciones entre granjas, p.ej. por movimiento de animals, y entre el ganado y la fauna salvaje. Además, la posibilidad de las autoridades nacionales de obtener datos moleculares de un vistazo mejora la colaboración con las instituciones de Salud Pública.

Fruto de este trabajo es el siguiente artículo científico:

- Rodriguez-Campos, S., S. González, L. de Juan, B. Romero, J. Bezos, C. Casal, J. Álvarez, I. G. Fernández-de-Mera, E. Castellanos, A. Mateos, J. L. Sáez-Llorente, L. Domínguez, y A. Aranaz, y la Red Española de Vigilancia de la Tuberculosis en Animales. 2011. **A database for animal tuberculosis (mycoDB.es) within the context of the Spanish national programme for eradication of bovine tuberculosis** [Una base de datos de tuberculosis animal (mycoDB.es) en el contexto del programa español de erradicación de la tuberculosis bovina]. Infection Genetics and Evolution. En prensa.

Asimismo, se presentaron las siguientes contribuciones en congresos y reuniones de proyectos europeos:

- Rodríguez, S., **The national database of *Mycobacterium bovis* and *Mycobacterium caprae*. Use in epidemiological surveys** (La base de datos nacional de *Mycobacterium bovis* y *Mycobacterium caprae*. Su uso en studios epidemiológicos). Presentación oral. Reunión del subgrupo de la Task Force (EFSA). Sevilla (España), 14-15 Noviembre 2007.

- Rodríguez, S., B. Romero, L. de Juan, S. González, J. Bezos, J. Álvarez, E. Castellanos, F. Lozano, N. Moya, N. Álvarez, T. Alende, A. Gutiérrez, F. Gallardo, A. Mateos, A. Aranaz y L. Domínguez. **The national database of Spanish *Mycobacterium bovis* and *Mycobacterium caprae* isolates** (La base de datos nacional de aislados españoles de *Mycobacterium bovis* y *Mycobacterium caprae*). Presentación oral. Taller "VNTR/MIRU and DVR-spoligotyping for *Mycobacterium bovis* typing. Results of the VNTR-DVR Spoligotyping Ring Trial." del Proyecto Europeo SSPE-CT-2004-501903. Madrid (España), 24-25 Marzo 2009.

- Romero, B., S. Rodríguez, J. Bezos, J. Álvarez, E. Castellanos, S. González, F. Lozano, N. Moya, A. Gutiérrez, T. Alende, J. L. Sáez, A. Mateos, A. Aranaz y L. Domínguez. **Spanish database of animal mycobacteriosis.** (Base de datos española de micobacteriosis en animales). Póster. Final meeting European project SSPE-CT-2004-501903. Turín (Italy), 17-19 Junio 2009.

- de Juan, L., S. Rodríguez, B. Romero, A. Aranaz, J. Bezos, E. Castellanos, S. González, J. L. Sáez, A. Mateos y L. Domínguez. **Spanish database of animal mycobacteriosis (mycoDB): application in epidemiological studies.** [Base de datos española de micobacteriosis (mycoDB): aplicación en estudios epidemiológicos]. Oral presentation. 31st Annual Congress of the European Society of Mycobacteriology. Bled (Slovenia), 4-7 Junio 2010.

Capítulo IV: La filogenia de *M. bovis* en la Península Ibérica

La filogenia global de *M. tuberculosis* es tratada en varios estudios acerca de los linajes de *M. tuberculosis* y su distribución geográfica, pero la filogenia de *M. bovis* fue apenas comprendida hasta hace poco. El objetivo de este capítulo fue el estudio de aislados españoles en relación a dos complejos clonales de *M. bovis* y el análisis del

ancestor común más reciente (MRCA) de la población española de *M. bovis*. El objetivo del estudio del MRCA fue la identificación de un marcador filogenético para aislados españoles de *M. bovis* y la evaluación de su presencia en Europa. Debido a la estrecha relación entre las poblaciones de *M. bovis* en España y Portugal el estudio fue ampliado a la Península Ibérica.

Como resultado de esta investigación se publicó el siguiente artículo científico:

- Rodriguez-Campos, S., A. C. Schürch, J. Dale, A. J. Lohan, M. V. Cunha, A. Botelho, K. De Cruz, M. L. Boschiroli, M. B. Boniotti, M. Pacciarini, M. C. Garcia-Pelayo, B. Romero, L. de Juan, L. Domínguez, S. V. Gordon, D. van Soolingen, B. Loftus, S. Berg, R. G. Hewinson, A. Aranaz y N. H. Smith. **European 2 – a clonal complex of *Mycobacterium bovis* dominant in the Iberian Peninsula** (European 2 - un complejo clonal de *Mycobacterium bovis* dominante en la Península Ibérica). Infection, Genetics and Evolution. En prensa.

Además, derivaron las siguientes colaboraciones en dos artículos científicos de este estudio:

- Berg, S., M. C. Garcia-Pelayo, B. Müller, E. Hailu, B. Asiimwe, K. Kremer, J. Dale, M. B. Boniotti, S. Rodríguez, M. Hilty, L. Rigouts, R. Firdessa, A. Machado, C. Mucavele, B. Nare Ngandolo, J. Bruchfeld, L. Boschiroli, A. Müller, N. Sahraoui, M. Pacciarini, S. Cadmus, M. Joloba, D. van Soolingen, A. L. Michel, B. Djønne, A. Aranaz, J. Zinsstag, P. van Helden, F. Portaels, R. Kazwala, G. Källenius, R. G. Hewinson, A. Aseffa, S. V. Gordon y N. H. Smith. 2011. **African 2, a clonal complex of *Mycobacterium bovis* epidemiologically important in East Africa** (African 2, un complejo clonal de Mycobacterium bovis epidemiológicamente importante en África del Este). Journal of Bacteriology 193:670-678.

- Smith, N. H., S. Berg, J. Dale, A. Allen, S. Rodríguez, B. Romero, F. Matos, S. Ghebremichael, C. Karoui, C. Donati, A. da Conceicao Machado, C. Mucavele, R. R. Kazwala, M. Hilty, S. Cadmus, B. N. R. Ngandolo , M. Habtamu, J. Oloya, A. Müller, F. Milian-Suazo, O. Andrievskaia, M. Projahn, S. Barandiarán, A. Macías, B. Müller, M. Santos Zanini, C. Y. Ikuta, C. A. Rosales Rodriguez, S. R. Pinheiro, A. Figueroa, S. N. Cho, N. Mosavari, P. N.Chuang, J. Zinsstag, D. van Soolingen, E. Costello, A. Aseffa, F. Proaño-Perez, F. Portaels, L. Rigouts, A. A.Cataldi, D. M. Collins, M. L. Boschiroli, R. G. Hewinson, J. S. Ferreira Neto, Om Surujballi, K. Tadyon, A. Botelho, A. M. Zárraga, N. Buller, R. Skuce, R. Jou, A. Michel, A. Aranaz, B.-Y. Jeon, G. Källenius, S. Niemann, M. B. Boniotti, P. D. van Helden, B. Harris, M. J. Zumárraga y K. Kremer. 2011. **European 1: A globally important clonal complex of *Mycobacterium bovis*** (European 1: Un complejo clonal de *Mycobacterium bovis* importante a nivel global). Infection, Genetics and Evolution 11:1340-1351.

En conjunto, los cuatro capítulos de esta memoria incluyen ocho artículos publicados, tres de ellos resultado de colaboraciones con otras instituciones. Además, se

incluye un manuscrito listo para enviar: “Discrimination of variable number repeat typing rises with the expansion of a clonal group of *Mycobacterium bovis* (La discriminación por análisis del número de repeticiones en tándem aumenta con la expansión de un grupo clonal de *Mycobacterium bovis*)”. En el capítulo II se incluye un estudio por espoligotipado y análisis del número de repeticiones en tándem en una selección de aislados de ganado de lidia cuyo manuscrito se encuentra en preparación. Parte de la introducción de la memoria sera enviada como revisión a una revista internacional con el título: “An update of molecular typing of *Mycobacterium bovis* and *Mycobacterium caprae* and its relevance for epidemiological studies (Una actualización de la tipificación de *Mycobacterium bovis* y *Mycobacterium caprae* y su importancia en estudios epidemiológicos)”.

Resumen

La tuberculosis es una zoonosis de origen bacteriano que representa un importante problema tanto en las especies de abasto como en animales salvajes, así como para la salud pública. *Mycobacterium* (*M.*) *bovis* y *M. caprae* son las especies que mayor importancia tienen en la tuberculosis en animales en España. Ambas se agrupan en el taxón "complejo *M. tuberculosis*" junto con *M. tuberculosis sensu stricto* (afecta principalmente a humanos), *M. africanum* (grupo heterogéneo de cepas principalmente de África ecuatorial), *M. bovis* BCG (cepas vacunales derivadas de *M. bovis*), *M. microti* (afecta principalmente a roedores), *M. canettii* (cepa africana) y *M. pinnipedii* (afecta principalmente a pinnípedos). En animales de abasto, la tuberculosis da lugar a importantes pérdidas económicas debidas a gastos generados en la erradicación, compensaciones a los propietarios por el sacrificio obligatorio (en los casos de tuberculosis bovina y determinados casos de tuberculosis caprina), al descenso en la producción y el aprovechamiento de nutrientes y decaimiento progresivo y a las limitaciones al comercio de los animales y sus productos a las que dan lugar (Steele, 1995; Aranaz *et al.*, 1999; Bennett y Cooke, 2006; Boland *et al.*, 2010; Bezos *et al.*, 2011). La infección en animales salvajes es importante puesto que éstos representan un reservorio de la tuberculosis, y la infección en especies protegidas y en peligro de extinción conlleva un aumento de la morbididad y mortalidad (Briones *et al.*, 2000; Peña *et al.*, 2006; Gortázar *et al.*, 2008; OIE, 2009). *M. bovis* y *M. caprae* también constituyen un peligro para la salud pública, sobre todo asociados al ámbito veterinario y ganadero, pero también al consumo de productos lácteos sin pasteurizar (Gutiérrez *et al.*, 1997; Grange, 2001; Prodinger *et al.*, 2002b; LoBue *et al.*, 2003; Winter *et al.*, 2005; de la Rua-Domenech, 2006; Rodwell *et al.*, 2008). A la luz de la legislación europea, nacional y autonómica, que restringe la comercialización y el movimiento de productos y animales procedentes de los rebaños infectados, la erradicación de la tuberculosis bovina es un objetivo importante para todos los países. Desde su implementación a finales de los años 90, los estudios de epidemiología forman uno de los pilares de los programas de erradicación. El diagnóstico basado en la caracterización molecular permite un mejor entendimiento de factores como la transmisión entre ganado vacuno y el papel de los animales salvajes además de otros factores medioambientales (Durr et al., 2000; Neill et al., 2005). Las técnicas más usadas actualmente son el espoligotipado (*direct variable repeat spacer oligonucleotide typing*, DVR-spoligotyping) (Kamerbeek *et al.*, 1997) y también el análisis de unidades de repetición intercaladas en el genoma de las micobacterias (*mycobacterial interspersed repetitive unit*, MIRU) y del número variable de repeticiones en tándem (*variable number tandem repeat*, VNTR) (Frothingham y Meeker-O'Connell, 1998; Supply *et al.*, 2000).

En la presente memoria evaluamos la epidemiología molecular en España mediante la aplicación de las técnicas de caracterización molecular espoligotipado y análisis por MIRU-VNTR, apoyado con el empleo de microarray de ADN, secuenciación de genoma completo y tipificación de polimorfismos de un único nucleótido (SNP), y analizamos los resultados en el contexto europeo. Para ello se han realizado varios estudios englobados en los cuatro capítulos de esta tesis doctoral.

En el primer capítulo tratamos el espoligotipado en cepas de *M. bovis* y *M. caprae*. La técnica del espoligotipado se dirige al *locus* de repetición directa (*direct repeat*, DR) que está compuesto por unas secuencias repetidas denominadas DR e intercaladas por otras secuencias polimórficas llamadas espaciadores; una unidad de DR y espaciador adyacente se denomina DVR (*direct variant repeat*) (Kamerbeek *et al.*, 1997; van Embden *et al.*, 2000). Una gran ventaja de esta técnica es la existencia de una base de datos internacional disponible desde 2003 en la página web www.mbovis.org (Smith y Upton, 2011) que posibilita la comparación de espoligotipos de diferentes estudios. Esta técnica se ha utilizado exhaustivamente para tipificar cepas españolas (Aranaz *et al.*, 1996; Gutiérrez *et al.*, 1997; Parra *et al.*, 2003; Gortázar *et al.*, 2005) y los resultados indicaron una gran diversidad, mayor que en estudios de poblaciones realizados en otros países. Para evaluar la situación epidemiológica en cuanto a la distribución de aislados de *M. bovis* y *M. caprae* en España en las diferentes Comunidades Autónomas, regiones geográficas y especies de animales infectadas, se llevaron a cabo dos estudios incluyendo los resultados de espoligotipado de 6215 aislados de *M. bovis* (de 1992 a 2007) y 791 aislados de *M. caprae* (de 1992 a 2009).

M. bovis se encontró en todo el territorio nacional, excepto en las Comunidades Autónomas de Murcia, Valencia, Ceuta y Melilla, e infectó tres especies de animales domésticos (ganado bovino, caprino y porcino), siete especies de animales salvajes (jabalí, ciervo, gamo, lince ibérico, zorro, rebeco y tejón), animales de compañía (gato y perro) y un animal de zoo (muflón). El espoligotipado resultó en 252 perfiles alcanzando un índice discriminatorio de 0.87 (Hunter y Gaston, 1988; Hunter, 1990). Semejante diversidad de espoligotipos es inusual en otros países como Gran Bretaña (Hewinson *et al.*, 2006), Irlanda del Norte (Skuce *et al.*, 2005) y Australia (Cousins *et al.*, 1998). Con respecto a Europa continental existen rasgos comunes entre los perfiles de espoligotipo y su diversidad entre las poblaciones de *M. bovis* de España, Francia, Portugal e Italia (Haddad *et al.*, 2001; Duarte *et al.*, 2008; Boniotti *et al.*, 2009). Por ejemplo, SB0121, el espoligotipo más frecuente en España (27.9%), también es el más abundante en Portugal y está presente, aunque con menos frecuencia, en Francia e Italia. El espoligotipo SB0121 se diferencia del espoligotipo con perfil de la cepa vacunal BCG, llamado *BCG-like* que es el más abundante en Francia e Italia, en la ausencia del espaciador 21. La pérdida del espaciador 21 es una característica que presenta el 67.1%

de los aislados de *M. bovis* españoles. Aún se desconocen las razones que conducen a la abundancia de las cepas de tipo SB0121. Podría tener una ventaja evolutiva sobre otras cepas, aunque la técnica del espoligotipado podría carecer de poder discriminatorio para diferenciar estas cepas. En cuanto a las diferentes especies de animales se encontró la mayor diversidad en el ganado bovino con 239 patrones de espoligotipo de los cuales 207 se encontraron unicamente en el ganado vacuno. En general, los espoligotipos más frecuentes estuvieron presentes tanto en animales domésticos como en fauna salvaje y ditribuidos por todo el país, exceptuando tres espoligotipos (SB0135, SB1232 y SB1258) obtenidos de muestreos intensivos en regiones delimitadas y en periodos de tiempo cortos. Concluimos que el espoligotipado es una herramienta útil para el estudio de la epidemiología molecular de la infección por *M. bovis* en España, debido a la gran variedad existente.

M. caprae es el agente principal de la tuberculosis caprina en España, y aunque actualmente no existen datos oficiales sobre su prevalencia debido a la no existencia de un programa nacional. Su identificación inicial se basa en la ausencia de los espaciadores 1, 3 a 16, 28 y 39 a 43 del perfil de espoligotipo, no obstante, se recomienda la identificación molecular adicional mediante reacción en cadena de la polimerasa (PCR) dirigida a la región de diferencia (RD) 4 (Brosch *et al.*, 2002; Mostowy *et al.*, 2002). Por ello, en 63 cepas seleccionados de los 791 aislados de *M. caprae* se llevó a cabo la PCR con tres cebadores según Mostowy *et al.* (2002). Además, un aislado de cada patrón de espoligotipo fue estudiado mediante secuenciación del gen *pncA* completo y de parte del gen *gyrB* para determinar polimorfismos específicos de *M. caprae*. Aparte de esto, se obtuvieron datos no publicados mediante la secuenciación del fragmento 5' de la región DR (número de acceso GenBank Z48304) de cinco aislados de los tres espoligotipos de *M. caprae* más prevalentes en España [SB0157 (n=1), SB0416 (n=3) y SB1084 (n=1)]. Dicha región se amplificó con los cebadores DR681 y DR2525. El análisis de esta región mostró un gran nivel de homogeneidad puesto que los cinco aislados compartieron las deleciones de DVR 1, DVR 2, y DVR 4 a DVR26 [de acuerdo con la numeración de van Embden *et al.* (2000)] que incluye los espaciadores 1, y 3 a 16 de la membrana estándar en uso. Las deleciones comprenden exactamente el DR y el espaciador adyacente. Los DVRs 27, 28 y 30 (espaciadores 17, 18 y 20) estaban presentes en las cepas secuenciadas, mientras que los resultados para DVR29 (espaciador 19) y DVRs 31 a 34 (espaciadores 21 a 24) variaron. Un dato interesante a destacar es la ausencia de los espaciadores 30 a 33 en todos los aislados excepto SB0418 y SB1619, ambos aislados de ganado bovino importado del este de Europa.

La diversidad entre las cepas de *M. caprae* era baja comparada con la de *M. bovis*; 791 aislados de *M. caprae* se agruparon en 15 perfiles de espoligotipo (índice discriminatorio D=0.58). La mayoría de los aislados provenía de ganado caprino (68.5%),

pero también encontramos un número considerable de aislados de ganado bovino (28.9%) además de ganado ovino, porcino, jabalíes, ciervos y un zorro. La importancia de este patógeno en el ganado bovino se refleja en un incremento de infecciones por *M. caprae* estadísticamente significativo desde el año 2004. A pesar de observar más infecciones en ganado bovino en regiones con un censo caprino más alto, se pudo excluir contacto directo con pequeños rumiantes en la mayoría de explotaciones afectadas, lo que sugiere que este patógeno puede ser mantenido en la población bovina. Además de los estudios mencionados anteriormente, se realizó el análisis por MIRU-VNTR usando ocho marcadores (ETR-A, ETR-B, ETR-D, QUB11a, QUB11b, QUB3232, ETR-E y MIRU26) en una selección de 20 aislados proveniente de diez explotaciones en cada una de ellas co-existiendo dos espoligotipos diferentes. En el caso de cinco explotaciones el cambio de espoligotipo podría explicarse con un único evento de deleción, mientras el perfil de MIRU-VNTR permaneció igual. En conclusión, observamos una amplia distribución de *M. caprae* en el territorio nacional y un aumento de infecciones por ese agente en ganado bovino, y sugerimos una mayor atención y control de *M. caprae* sobre todo en países con un censo de ganado caprino importante.

Realizamos una revisión de los espoligotipos recogidos en www.mbovis.org, cuyo perfil indica que pertenecen a las especies (o ecotipos) *M. bovis*, *M. caprae*, *M. microti* o *M. pinnipedii* y que están publicados en revistas internacionales. La información está resumida en una tabla que está disponible como material adicional incluida en el disco compacto que acompaña esta tesis doctoral. Los perfiles de espoligotipo se han comparado a menudo utilizando métodos de agrupamiento jerárquico como el método de análisis de grupos pareados sin ponderar usando la media aritmética (*unweighted pair group method with arithmetic mean*, UPGMA) para visualizar relaciones entre diferentes perfiles. Este método no es óptimo debido a la unidireccionalidad del *locus* DR y la tendencia a homoplasias. Sin embargo, los árboles filogenéticos obtenidos mediante este método son capaces de revelar posibles relaciones entre espoligotipos agrupando perfiles con deleciones de espaciadores parecidas y por tanto facilitando el análisis de un gran número de datos. Usamos el método UPGMA en tres diferentes selecciones de espoligotipos encontrados en España: 1) espoligotipos de *M. bovis* con presencia del espaciador 21 en su perfil de espoligotipado, 2) espoligotipos de *M. bovis* con ausencia del espaciador 21 de su perfil de espoligotipado, y 3) espoligotipos de cepas de *M. caprae*. La comparación de los espoligotipos encontrados en España con los de otros países, reveló ciertas características específicas, conocidas como huellas de espoligotipo (*spoligotype signature*) (Streicher et al., 2007). Singularmente en el caso de las cepas aisladas en la Península Ibérica encontramos una mayor abundancia de cepas de *M. bovis* con falta de espaciador 21 en su perfil de espoligotipo en la Península Ibérica, y la falta de los espaciadores 30 a 33 en el patrón de espoligotipo.

El desarrollo y la aplicación a gran escala de técnicas de genotipado como el espoligotipado han reforzado el control de la tuberculosis bovina. Sin embargo, en ciertos países o regiones geográficas el espoligotipado no ofrece una discriminación satisfactoria y es necesario complementarlo con el tipado por MIRU-VNTR (Skuce *et al.*, 2005; Hewinson *et al.*, 2006; Smith *et al.*, 2006). El análisis mediante MIRU-VNTR está dirigido a diferentes *loci* distribuidos por todo el genoma para aumentar el poder discriminatorio y permitir la trazabilidad de posibles fuentes de infección.

Mientras que el sector de salud pública trabajando con *M. tuberculosis* ha alcanzado un consenso sobre la combinación de *loci* para la tipificación por MIRU-VNTR, el uso de los diferentes marcadores para la caracterización de *M. bovis* está sometido a discusión. Una combinación de seis marcadores (ETR-A, ETR-B, ETR-D, QUB11a, QUB11b y QUB3232) fue propuesta por VENoMYC (acción europea coordinada SSPE-CT-2004-501903; Supply, 2006), pero la diversidad alélica, y por ende el poder discriminatorio, de los *loci* varía entre los países y por el momento se apuesta por combinaciones de *loci* diferentes. En España, además de los seis marcadores mencionados, se han elegido otros tres para el tipado de cepas de *M. bovis* [ETR-E, MIRU26 (Gortázar *et al.*, 2005); QUB26 (Romero *et al.*, 2008)]. Con el fin de profundizar nuestro conocimiento de las relaciones entre cepas españolas de *M. bovis*, el grado de diversidad en cuanto a la tipificación por MIRU-VNTR y para explotar esta técnica para el seguimiento de brotes de tuberculosis, hemos analizado tres paneles de aislados de *M. bovis* utilizando el conjunto de nueve marcadores de MIRU-VNTR. Los tres estudios están comprendidos en el segundo capítulo de la memoria.

El primer estudio por MIRU-VNTR usando nueve *loci* se dirigió a 115 aislados de *M. bovis* con espoligotipo SB0121, el más frecuente en España. El tipado resultó en 65 perfiles diferentes alcanzando un índice de discriminación (D) de 0.9856. Encontramos una expansión de un grupo de cepas estrechamente relacionadas. Incluso con una reducción de los *loci* seleccionados a los cuatro más discriminatorios (ETR-A, ETR-B, QUB11a y QUB3232) se mantuvo esa expansión, aunque con sólo 51 MIRU-VNTR tipos y una D algo más baja (0.9676). A diferencia de lo descrito en el Reino Unido (Smith *et al.*, 2006), no encontramos agrupamientos por regiones geográficas. El grado de diversidad observado en las cepas con espoligotipo SB0121 es congruente con resultados de Portugal (Cunha *et al.*, 2011) corroborando la estrecha relación entre las cepas de la Península Ibérica.

El segundo estudio tuvo como objetivo la identificación del genotipo que causó el primer brote de tuberculosis en alpacas en España. Los aislados, que procedieron de tres animales, mostraron el mismo genotipo [espoligotipo SB0295, MIRU-VNTR tipo 6-4-3-4-5-11-2-5-6 (en el siguiente orden: ETR-A, ETR-B, ETR-D, ETR-E,

MIRU26, QUB11a, QUB11b, QUB26 y QUB3232); la amplificación del *locus* QUB11a falló en uno de los aislados]. A continuación seleccionamos aislados de *M. bovis* posiblemente relacionados en el área geográfica alrededor comprendida en un radio de aproximadamente 150 km de las dos explotaciones de alpacas infectadas. Estos 47 aislados con espoligotipo SB0295 o espoligotipos relacionados, SB0121 y SB1190, se sometieron al análisis por MIRU-VNTR usando los nueve *loci*. Ninguno de los once perfiles identificados fue idéntico al perfil de la cepa que causó la tuberculosis en las alpacas. No obstante, como en el caso anterior, los genotipos estuvieron estrechamente relacionados, agrupándose en un grupo clonal y un subgrupo conectado, a excepción de cinco perfiles denominados *singletons* (únicos) que comparados con los demás perfiles variaron en más de un *locus*.

El tercer panel estudiado estaba compuesto por 39 aislados de *M. bovis* cultivados a partir de muestras de ganado de lidia y animales cinegéticos cazados en la misma finca. Los resultados obtenidos mediante espoligotipado y tipificación por MIRU-VNTR dirigido a los nueve *loci* resaltan la circulación de los espoligotipos más frecuentes en España en el ganado de lidia y la fauna salvaje que comparte hábitat. Además, se identificó un genotipo dominante en la seleccíon de cepas [espoligotipo: SB0295, MIRU-VNTR tipo: 6-4-3-3-5-10-2-5-7 (en el órden arriba indicado)] y siete espoligotipos únicos que podrían haber evolucionado de los tipos más frecuentes en un evento de deleción.

A lo largo de estos estudios se utilizó la cepa vacunal *M. bovis* BCG Danish (CCUG 27863, Colección de Cultivos de la Universidad de Gotemburgo, Suecia) como control positivo y los nueve *loci* fueron secuenciados (Centro de Investigaciones Biológicas, CIB, Madrid) para elaborar una tabla de asignación de alelos en acuerdo con las secuencias obtenidas y publicaciones existentes (Frothingham y Meeker O'Connell., 1998; Supply *et al.*, 2000; Roring *et al.*, 2002; Skuce *et al.*, 2002). Concluimos que entre las cepas españolas de *M. bovis* existe una gran variedad de MIRU-VNTR tipos que se expanden como grupos clonales de cepas relacionadas. Por lo tanto, aconsejamos una interpretación menos severa a la hora de identificar posibles fuentes de infección, considerando perfiles con variaciones de un solo alelo en un único *locus* o dos *loci*.

Dada la importancia de la caracterización molecular en el estudio epidemiológico el Ministerio de de Medio Ambiente, y Medio Rural y Marino decidió cetnralizar los registros de tuberculosis en animales en una base de datos nacional descrita en el tercer capítulo. Esta tarea fue encomendada al Centro de Vigilancia Sanitaria en Veterinaria (VISAVET) y una base de datos llamada mycoDB.es fue diseñada y puesta a punto dentro del proyecto de esta tesis doctoral. Cabe destacar que la colección de muestras, datos epidemiológicos y resultados de caracterización molecular no habría sido posible sin la entrega profesional de un gran número de compañeros, lo

cual refleja el compromiso en el estudio de la tuberculosis en animales a nivel nacional. Dicha base de datos constituye una herramienta importante en el programa nacional de erradicación de la tuberculosis bovina y el acceso se realiza a través de la página web de la Red de Alerta Sanitaria (RASVE) y debido a la ley de protección de datos es restringido a los Servicios Veterinarios y laboratorios que participan en el programa nacional. Una demostración de mycoDB.es de libre acceso, que contiene datos simulados, está disponible en www.mycoDB.es.

El acceso a una gran cantidad de datos moleculares sin duda mejora la comprensión de la epidemiología de esta enfermedad, pero conlleva el problema de almacenaje y visualización de los datos. La base de datos mycoDB.es está complementada con un visor geográfico que muestra la distribución geográfica de los aislamientos a nivel de municipio en mapas y ofrece la información sobre los espoligotipos, y en menor medida MIRU-VNTR tipos, identificados según diferentes criterios de consulta: año de aislamiento de la cepa, especie animal, Comunidad Autónoma, provincia y municipio. Un manual resumiendo las diferentes funciones está disponible para los usuarios y mycoDB.es se actualiza periódicamente. En su comienzo, mycoDB.es sólo centralizaba datos de espoligotipado desde el año 1996 hasta la actualidad con un número de aislados cada vez más grande (16230 aislados *M. bovis*, 1449 de *M. caprae* y 6 de *M. tuberculosis*, consultado el 29 de noviembre de 2011). En 2011 mycoDB.es fue rediseñada y ampliada a registros de MIRU-VNTR tipos para 357 aislados de *M. bovis*, subtipados en 129 MIRU-VNTR tipos diferentes, y 21 aislados de *M. caprae*, subtipados en 15 MIRU-VNTR tipos (consultado el 19 de septiembre 2011). La base de datos permite el acceso a los datos a través de cuatro opciones de búsqueda denominadas *Spoligotype Search* (por espoligotipo), *MIRU-VNTRtype Search* (por MIRU-VNTR tipo), *Isolate Search* (seleccionando diferentes criterios como localización, especie animal, genotipo, año de aislamiento o periodo de tiempo) e *Isolate maps* (mapas que muestran los aislamientos positivos anuales). La base de datos nacional mycoDB.es es una herramienta para la investigación de la epidemiología de la tuberculosis en animales que apoya al programa actual de erradicación de la tuberculosis bovina en España.

En el último capítulo comprendido en esta memoria se hace uso de métodos moleculares distintas a las técnicas de tipificación estándares para el estudio de la filogenia de *M. bovis* en la Península Ibérica. La estructura de la población del complejo *M. tuberculosis* es altamente clonal porque no se han encontrado signos de transferencia y recombinación de secuencias cromosómicas entre cepas. En poblaciones clonales las características genéticas como polimorfismos de secuencia larga (*large sequence polymorphism*, LSP) y SNPs son transmitidas a todos los descendientes (Smith *et al.*, 2006). El *locus* DR de estudiarlo con fines epidemiológicos mediante el espoligotipado, puede ser útil para el estudio de genética de poblaciones gracias a su evolución

unidireccional basándose en la pérdida de un único espaciador o un bloque de espaciadores contiguos (Fang *et al.*, 1998; van Embden *et al.*, 2000). Por ende, las anteriormente mencionadas huellas de espoligotipo (Streicher *et al.*, 2007) pueden servir como indicadores de ciertos linajes del complejo *M. tuberculosis* (Kato-Maeda *et al.*, 2011; Smith *et al.*, 2011).

Los análisis de las poblaciones de *M. bovis* en Portugal (Duarte *et al.*, 2008) y España (Rodríguez *et al.*, 2010) han revelado una firma de espoligotipo marcada por la ausencia del espaciador 21 que es común en el 70% de los aislados de *M. bovis* de la Península Ibérica. Con el objetivo de identificar un posible marcador filogenético para aislados de *M. bovis* sin espaciador 21 en su perfil de espoligotipo, se llevaron a cabo microarrays de ADN en cuatro cepas españolas sin el espaciador en cuestión, pero no se identificó ningún LSP que constituyera un marcador filogenético apto. A continuación, se eligieron tres cepas de *M. bovis* con la misma característica para secuenciación del genoma completo y analizamos los SNPs en común de las tres cepas que además estuvieron ausentes en las cepas de referencia *M. bovis* AF2122/97, *M. bovis* BCG y *M. tuberculosis* H37Rv. Identificamos un total de 108 SNPs de los cuales seleccionamos 16 para análizarlas en diez aislados españoles de *M. bovis*. Un SNP en el nucléotido con posición 3765573 (con respecto a *M. bovis* AF2122/97) del gen *guaA* era capaz de diferenciar las cepas sin espaciador 21 en su perfil de espoligotipo de las que sí lo tienen. El cambio de nucleótido en este gen, que codifica una posible sintetasa de guanina monofosfato, es sinónimo ocurriendo en un triplete codificando alanina. Para evitar la costosa secuenciación se puso a punto una PCR seguida de una restricción enzimática (*PCR-restriction endonuclease analysis*, PCR-REA) para determinar la presencia de este SNP en paneles representativos de aislados de *M. bovis* de España (n=201), Portugal (n=48), Francia (n=145) e Italia (n=50). La ausencia junto con el SNP en *guaA* define un complejo clonal de *M. bovis*, denominado European 2 (Eu2), que es predominante en la Península Ibérica, poco frecuente en Francia e Italia y ausente de las Islas Británicas.

M. bovis está presente en la mayoría de países africanos, y actualmente dos complejos clonales se han descrito en el continente africano, el African 1 (Müller *et al.*, 2009) y el African 2 (Af2) (Berg *et al.*, 2011). En África del Este se ha observado una huella de espoligotipo prevalente marcada por la ausencia de los espaciadores 3 a 7 y mediante microarray de ADN se ha identificado una deleción de 14.1 kb que comprende el operón *mce2*, llamada RDAf2. La ausencia o presencia de RDAf2 se determina mediante PCR. La combinación de la ausencia de espaciadores 3 a 7 del patrón de espoligotipo y la deleción de RDAf2 define el complejo clonal Af2, prevalente en Uganda, Burundi, Tanzanía y Etiopía. Entre los diferentes perfiles de espoligotipo en España también se encuentran algunos con la huella de espoligotipo característica de este

complejo clonal Af2. Con el fin de estimar la proporción de aislados españoles de *M. bovis* pertenecientes a este linaje, un panel seleccionado mediante PCR dirigida a la Región de Diferencia (RD) Af2. En España menos del 1% de los aislados de *M. bovis* presentan la huella de espoligotipo típica de cepas Af2 y el análisis de RDAf2 en 20 aislados con esta huella característica no reveló ningún miembro del complejo clonal Af2. Este trabajo forma parte de una colaboración entre 19 países de África y Europa (Berg *et al.*, 2011).

En el Reino Unido y la República de Irlanda la mayoría de aislados de *M. bovis* no presenta el espaciador 11 en su perfil de espoligotipo y una deleción, RD Eu1, fue identificada en cepas con esa firma de espoligotipo. Esa deleción, anteriormente conocida como RD17 (Gordon *et al.*, 2001), tiene 806 pb y está localizada en el gen *treY* que codifica una sintetasa de maltooligosiltrehalosa, una enzima importante en la biosíntesis del disacárido trehalosa (De Smet *et al.*, 2000). La deleción de RDEu1 junto con la ausencia del espaciador 11 del patrón de espoligotipo define un complejo clonal de *M. bovis*, denominado European 1 (Eu1), que comprende casi la totalidad de cepas en las Islas Británicas (Smith *et al.*, 2011). Aislados de *M. bovis* sin el espaciador 11 en su espoligotipo se encontraron en muchos países de diferentes continentes, y un estudio extensivo fue llevado a cabo para determinar la presencia global del *locus* RDEu1. Un total de 80 aislados de *M. bovis* de España fueron tipadas por PCR dirigida al RDEu1. Este análisis fue incluido en un trabajo de colaboración entre 28 países que describió la distribución del complejo clonal Eu1 a nivel global siendo la proporción máxima de cepas pertenecientes al complejo clonal Eu1 en España 6.1% (Smith *et al.*, 2011).

Consideramos que los estudios incluidos en la presente memoria han contribuido a la mejor comprensión de la epidemiología molecular de M. bovis y *M. caprae* en España, y por ende, han aportado conocimientos importantes para el programa nacional de erradicación de la tuberculosis bovina.

Conclusiones

1. El espoligotipado reveló un grado de diversidad alto (D=0.87) en los aislados españoles de *Mycobacterium bovis* y por tanto es una herramienta útil para el estudio de la epidemiología molecular de la infección en nuestro país. Sin embargo, los cinco espoligotipos más frecuentes de *M. bovis*, SB0121, SB0134, SB0339, SB0265 y SB0295, agrupan más del 50% de los aislados. Los espoligotipos más frecuentes están presentes en todo el territorio nacional, tanto en animales domésticos como en fauna salvaje.

2. La diversidad de las cepas de *Mycobacterium bovis* es más alta en el ganado vacuno que en otras especies de animales domésticos y salvajes, lo que indica que la enfermedad se mantiene en el ganado vacuno. La diversidad de cepas también varía entre regiones geográficas, y parece no estar afectada por la política de erradicación.

3. *Mycobacterium caprae* es el agente etiológico de la tuberculosis caprina que está extendida en España. También constituye un riesgo para la salud de otros animales domésticos, fauna salvaje y humanos. El espoligotipo más frecuente de *M. caprae*, SB0157, agrupa más del 40% de los aislados.

4. Los casos de tuberculosis bovina causada por *Mycobacterium caprae* han aumentado significativamente durante los últimos años. Aunque esto sugiere que el patógeno es capaz de circular en y entre explotaciones de ganado vacuno, sería importante considerar el inicio de un programa de erradicación en el ganado caprino, puesto que es el principal reservorio de este patógeno.

5. Los aislados de *Mycobacterium caprae* de España muestran una huella de espoligotipo específica con la falta de los espaciadores 30 a 33 en su patrón de espoligotipado, al contrario de cepas de *M. caprae* de Europa Central y del Este. La existencia de una característica especial del grupo ibérico de *M. caprae* facilita la identificación de brotes en Europa.

6. El espoligotipo de *Mycobacterium bovis* más frecuente en España, SB0121, presenta una gran diversidad de tipos de unidades de repetición dispersas en las micobacterias-número variable de repeticiones en tándem (MIRU-VNTR tipos) y se expande como un conjunto de grupos clonales estrechamente relacionados.

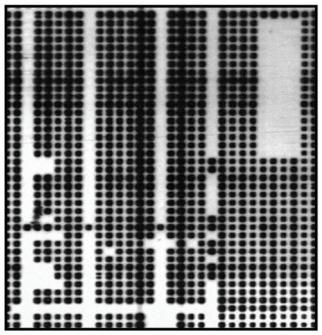
7. Debido a la gran diversidad de la población española de *Mycobacterium bovis*, podría aplicarse una interpretación menos severa del análisis mediante unidades

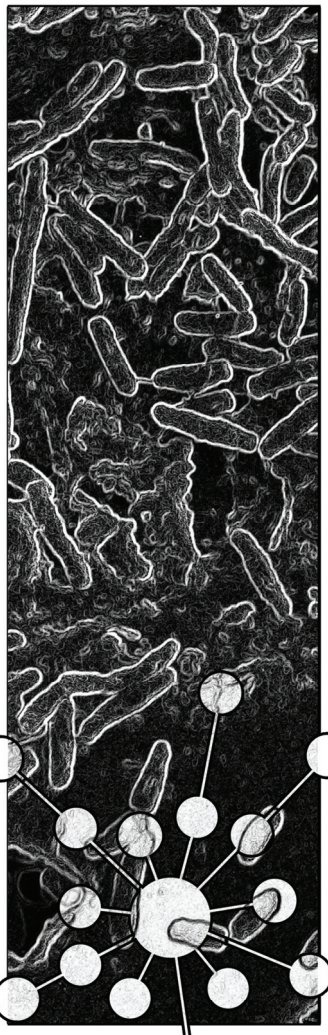
de repetición dispersas en las micobacterias-número variable de repeticiones en tándem (MIRU-VNTR) cuando se trata de identificar posibles fuentes de brotes.

8. La combinación de los cuatro *loci* ETR-A, ETR-B, QUB11a y QUB3232 constituye un panel de marcadores de unidades de repetición dispersas en las micobacterias-número variable de repeticiones en tándem (MIRU-VNTR) recomendable para su uso en cepas españolas de *Mycobacterium bovis*. Para estudios más específicos pueden analizarse adicionalmente: ETR-D, ETR-E, MIRU26, QUB11b y QUB26.

9. La base de datos nacional mycoDB.es es una herramienta valiosa para la epidemiología de la tuberculosis en animales y forma uno de los pilares del programa actual de erradicación de la tuberculosis bovina en España.

10. La pérdida del espaciador 21 del patrón de espoligotipo, junto con un cambio en un nucleótido en el gen *guaA*, caracteriza el complejo clonal European 2 (Eu2) de *Mycobacterium bovis*, que es prevalente en la Península Ibérica. En España, aproximadamente el 70% de la población de *M. bovis* pertenece al complejo clonal Eu2, mientras los miembros de este linaje son menos frecuentes en Francia e Italia, y ausente en las Islas Británicas. Los miembros del complejo clonal Eu1 son poco frecuentes en España, y no se ha identificado ningún miembro del complejo clonal African 2.





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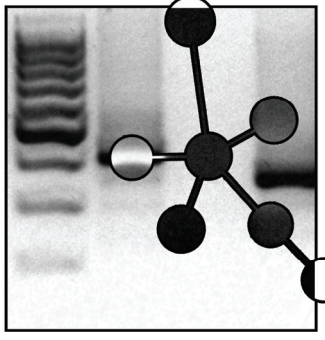

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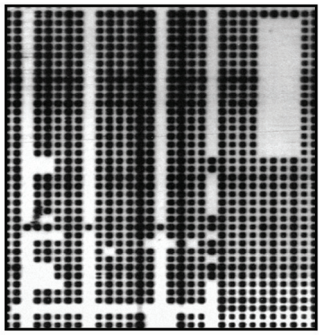
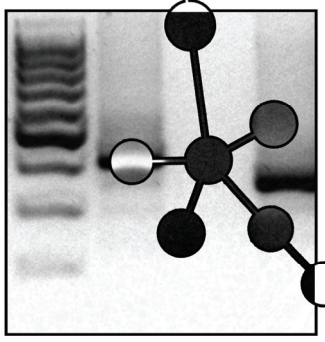


Appendix



Appendix I - List of abbreviations

A: Adenine

BC: Before Christ

BP: Before present, referring to the time scale used in archaeology

C: Cytosine

CGH: Comparative genomic hybridization

CRISPR: Clustered regularly interspersed palindromic repeat

DR: Direct repeat

DVR: Direct variant repeat

EFSA: European Food Safety Authority

ELISA: Enzyme-linked immunosorbent assay

ETR: Exact tandem repeat

EU: European Union

G: Guanine

gyrA: Gyrase A gene

gyrB: Gyrase B gene

HIV: Human immunodeficiency virus

HT-NGS: High throughput next-generation sequencing

IFN-γ: Interferon-gamma

IS: Insertion sequence

katG: Catalase-peroxidase gene

LJ: Löwenstein-Jensen

MARM: Ministerio de Medio Ambiente, y Medio Rural y Marino/Ministry of the Environment, and Rural and Marine Affairs

MIRU: Mycobacterial interspersed repetitive unit

MLST: Multilocus sequence typing

mpt40: Membrane protein 40

MPTR: Major polymorphic tandem repeat

MS: Member State of the European Union

MTBC: *Mycobacterium tuberculosis* complex

OIE: Office Internationale des Epizooties/World Organisation for Animal Health

ORF: Open reading frame

OTF: Officially tuberculosis free

PCR: Polymerase chain reaction

PFGE: Pulsed field gel electrophoresis

pncA: Pyrazinamidase gene

PPD: Purified protein derivative

PZA: Pyrazinamide

QUB: Queen's University Belfast

RAPD: Random amplified polymorphic deoxyribonucleic acid

RD: Region of difference

REA: Restriction endonuclease analysis

RFLP: Restriction fragment length polymorphism analysis

RoI: Republic of Ireland

SBS: Sequencing by synthesis

SMRT: Single molecule real time

SOLiD: Sequencing by oligo ligation and detection

ST: Sequence type

T: Thymine

TBOF: Officially tuberculosis-free bovine herd

TCH: Thiophene-2-carboxylic acid

UK: United Kingdom

USA: United States of America

VNTR: Variable number tandem repeat

WHO: World Health Organisation

Appendix II - List of figures

Figure 1 Phylogenetic tree of mycobacteria based on 16S rRNA sequence

Figure 2 Schematic representation of the cell envelope of *Mycobacterium tuberculosis*.

Figure 3 Circular representation of the *Mycobacterium tuberculosis* H37Rv genome

Figure 4 Circular representation of the *Mycobacterium bovis* genome

Figure 5 Genealogy of *Mycobacterium bovis* BCG strain dissemination

Figure 6 Evolution of the *Mycobacterium tuberculosis* complex based on regions of difference and single nucleotide polymorphisms

Figure 7 The phylogenetically informative mutations in the lineage leading to *Mycobacterium bovis*

Figure 8 Localisation of the *Mycobacterium bovis* Af1 and Af2 clonal complexes in Africa

Figure 9 European spread of agropastoralism

Figure 10 Global status of bovine tuberculosis

Figure 11 Herd prevalence and animal incidence of bovine tuberculosis in Spain

Figure 12 Distribution of the goat population in the European Union

Figure 13 Estimated incidence rates for human tuberculosis by country

Figure 14 Overview over the most important techniques used for typing of the *Mycobacterium tuberculosis* complex

Figure 15 Scheme of the direct repeat (DR) region and the spoligotyping technique

Figure 16 Scheme of variable number tandem repeat (VNTR) loci and VNTR typing

Figure 17 Dendrogram showing 164 *M. bovis* spoligotypes, with spacer 21 present in their pattern, isolated in Spain. The tree was built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

Figure 18 Dendrogram showing 259 spacer 21-deleted *M. bovis* spoligotypes isolated in Spain. The tree was built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

Figure 19 Dendrogram showing 15 *M. caprae* spoligotypes found in Spain. The tree was built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

Figure 20 Maps of Spain showing (A) the provinces with most bullfighting farms and the municipalities of the farms included in this study, and (B) the herd prevalence of bovine tuberculosis by veterinary district

Figure 21 Welcome page of the Spanish database for animal tuberculosis mycoDB.es

Appendix III - List of tables

Table 1 Cultural and biochemical characteristics for the *M. tuberculosis* complex

Table 2 Overview of molecular characteristics for the *M. tuberculosis* complex

Table 3 Herd prevalence of bovine tuberculosis in the Autonomous Communities of Spain from 2005-2010

Table 4 Published whole genome sequences of mycobacterial species

Table 5 Spoligotype patterns for selected members of the *Mycobacterium tuberculosis* complex

Table 6 Spoligotyping data from population surveys conducted in Europe and overseas

Table 7 Discriminatory variable number tandem repeat (VNTR) markers for typing of members of the *M. tuberculosis* complex

Table 8 Allele calling table for the nine VNTR loci used for the VNTR typing studies. The results for the positive control at each locus is shown

Table 9 Allelic diversity of the individual MIRU-VNTR markers and discriminatory index of spoligotyping and MIRU-VNTR typing and different combinations of these for the set of 39 isolates of *M. bovis* from bullfighting cattle

Table 10 Presence of spoligotypes from other domestic or wild animal species in the same municipality or province of the 16 farms with bullfighting cattle

Table 11 Spanish *Mycobacterium bovis* strains deletion typed for RDAf2. Extract from the supplementary material

Table 12 Spanish *Mycobacterium bovis* strains deletion typed for RDEu1. Extract from the supplementary material

Appendix IV - Sequence submitted to GenBank

Mycobacterium bovis strain MI06/05041 GMP synthase (*guaA*) gene, partial cds

GenBank: JF920303.1

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SEP-2011
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VERSION     JF920303.1  GI:334725242
KEYWORDS    .
SOURCE      Mycobacterium bovis
ORGANISM    Mycobacterium bovis
            Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
            Corynebacterineae; Mycobacteriaceae; Mycobacterium;
            Mycobacterium tuberculosis complex.
REFERENCE   1  (bases 1 to 567)
  AUTHORS   Rodriguez-Campos,S., Schurch,A.C., Dale,J., Lohan,A.J.,
            Cunha,M.V., Botelho,A., De Cruz,K., Boschiroli,M.L.,
            Boniotti,B., Pacciarini,M., Garcia-Pelayo,M.C., Romero,B.,
            de Juan,L., Dominguez,L., Gordon,S.V., van Soolingen,D.,
            Loftus,B., Berg,S., Hewinson,R.G., Aranaz,A. and Smith,N.H.
  TITLE     European 2 - a clonal complex of Mycobacterium bovis
            dominant in the Iberian Peninsula
  JOURNAL   Infect. Genet. Evol. (2011) In press
REFERENCE   2  (bases 1 to 567)
  AUTHORS   Rodriguez-Campos,S., Schurch,A.C., Aranaz,A. and Smith,N.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAY-2011) Departamento de Sanidad Animal,
            Facultad de Veterinaria, and Centro Visavet, Universidad
            Complutense Madrid,Spain, Avenida Puerta de Hierro s/n,
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      541 cgcggccgtg accgcgtcac cgtgact
//
```

Appendix V - Review of published spoligotypes

The table resuming the review of the literature on spoligotypes with *M. bovis*-, *M. caprae*-, *M. microti* and *M. pinnipedii*-like patterns included in the international database www.mbovis.org (Smith and Upton, 2011) is provided in electronic format.

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The thesis "Molecular epidemiology of *Mycobacterium bovis* and *Mycobacterium caprae* in Spain" includes large-scale studies of *M. bovis* and *M. caprae* using the standard typing techniques spacer oligonucleotide typing and variable number tandem repeat typing to assess the diversity of the Spanish strains. All the molecular data on animal tuberculosis at national level are collected in a database to facilitate epidemiological studies. Furthermore, these techniques were applied in the study of tuberculosis outbreaks caused by *M. bovis* to disclose the clonal expansion of groups of related strains. Additionally, the phylogeny of *M. bovis* in the Iberian Peninsula was studied by DNA microarrays and whole genome sequencing. A clonal complex, European 2, was defined by a spoligotype signature plus a single nucleotide polymorphism. The distribution of this clonal complex in Europe was determined.

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